Neth. J. Pl. Path. 86 (1980) 79–98

Spinach latent virus, a new ilarvirus seed-borne in Spinacia oleracea

L. BOS, H. HUTTINGA and D. Z. MAAT

Research Institute for Plant Protection (IPO), Wageningen

Accepted 3 July 1979

Abstract

During 1977 and 1978 an apparently new virus was isolated from samples of 12 out of 142 lots of spinach seed from a number of countries which did not produce symptoms in spinach. In one sample seed infection was over 50%. The virus was later found to be identical with a virus encoded GE36, earlier presumed to have been isolated from apple and pear (Van der Meer, 1968; Maat and Vink, 1971). It has now been further characterized and named spinach latent virus.

Seventeen out of 36 plant species tested were susceptible, most without producing symptoms. *Myzus persicae* did not transmit the virus, but rates of seed transmission in artificial hosts were high: 53% in *Celosia cristata*, over 90% in *Chenopodium quinoa*, 30% in *Nicotiana rustica*, 72% in *N. tabacum* 'Samsun', 90% in 'White Burley' and 94% in 'Xanthi'. Infection is in the embryo. The virus can also be detected in dry seeds.

Purified virus contained three components sedimenting at 87, 98 and 108 S. In the electron microscope the virus particles were irregularly spherical and c. 27 nm in diameter. They were hard to detect in crude plant sap. Some particles were bacilliform. The buoyant density in cesium sulphate was 1.269×10^3 kg.m^{-3}. Five RNA components were detected having relative molecular masses of 1.30, 1.18, 0.91, 0.35 and 0.27×10^6, respectively. To induce infection the three largest components are required plus the two smallest components or the coat protein. Molecular mass of the coat protein subunit was 28000. These characters are typical of ilarviruses.

The virus proved weakly immunogenic. An antiserum with a titre of 64 was produced. No serological differences could be found between the type isolate from spinach and GE36. The virus did not react with antisera to any of 36 spherical viruses and alfalfa mosaic virus.

Additional keyword: GE36 virus.

Introduction

An apparently unknown virus was detected in 1977 in commercial seed of spinach (*Spinacia oleracea*). The new virus was found to be present in many seed lots. High rates of seed transmission and reactions of test plants suggested a possible relationship to nepoviruses but the virus could not be identified with any of these. However, it appeared to be serologically related to the GE36 virus earlier supposed to have been isolated from apple and pear trees and studied in some detail in this laboratory by Van der Meer (1968) and Maat and Vink (1971).

The high incidence of the spinach virus in spinach justified the present detailed study and further comparisons with GE36 virus. Štefanac (1978) also isolated a seed- and

pollen-borne virus from spinach in Yugoslavia which she tentatively named spinach latent virus. It seems identical to the virus now fully described in this paper.

Materials and methods

Virus isolates. After initial detection in 1977 in spinach 'Securo' (Sp20–9) the virus was isolated from other Dutch and foreign seed samples. Since no obvious differences occurred between isolates, most research was on Sp20–9. The GE36 virus was isolated from seeds of *Chenopodium quinoa* obtained from Mr F. A. van der Meer, IPO.

Maintenance and propagation mostly was in *C. quinoa*, usually harvested 10 days after inoculation, and sometimes in symptomless plants of *Nicotiana rustica*. Long-term storage was in leaf material of *C. quinoa* dried and stored over $CaCl_2$ at 4°C, and in seeds of *C. quinoa*.

Biological studies were in the conventional ways, using *C. amaranticolor* as a local-lesion host and *C. quinoa* for its characteristic systemic reaction. Plants were grown in an insect-proof glasshouse at 18–22°C. Additional light from Philips SON/T lamps was given during short days to extend daylength to 12 h.

Host-range tests were with varying numbers of plants as indicated in Table 1. Back inoculations were made to at least two plants each of *C. amaranticolor* and *C. quinoa* from inoculated and non-inoculated leaves separately, at least 14 and 21 days after inoculation, respectively.

Seed testing mostly was by sowing seeds in flats, and testing five groups of ten seedlings each by sap inoculation onto two plants each of both *Chenopodium* species. Cucumber seedlings were often added so as to more readily detect contaminations with cucumber mosaic virus. In addition, all plants of rows that gave a positive reaction were then usually tested individually to more precisely determine percentage of seed transmission. For comparison, some spinach seed samples were also tested by grinding in water dry seeds and seeds soaked in water for one night, and subsequent inoculation onto test plants. Dry-seed testing was also used for *C. quinoa* and for *N. tabacum* 'Samsun' and 'Xanthi'.

Virus purification. Leaves from infected plants of *C. quinoa* with severe symptoms by Sp20–9 were stored overnight at 4°C. Portions of 100 g were then homogenized with a Waring blender in 300 ml of 18 mM phosphate-citric acid buffer, pH 7, (PCA buffer) containing 0.1% thioglycolic acid. The homogenate was filtered through two layers of cheesecloth and the filtrate was brought to 8% with *n*-butanol, stirred for 45 min and left for 30 min at 4°C. The mixture was then centrifuged for 10 min at 16000 *g* (all *g* values will be given at R_{max}). The upper phase was decanted and centrifuged for $2\frac{1}{2}$ h at 116000 *g*. The resulting pellets were resuspended in 20 ml of PCA buffer, stirred for $1\frac{1}{2}$ h at 4°C and the suspension was centrifuged for 10 min at 7700 *g*. The supernatant was centrifuged for $2\frac{1}{2}$ h at 116000 *g*. The pellets were resuspended in 1.5 ml of PCA buffer, stirred overnight at 4°C and the suspension was centrifuged for 10 min at 7700 *g*.

One ml portions of this suspension were each centrifuged for $2\frac{1}{2}$ h at 24000 rpm on a 10–40% sucrose gradient in a Beckman SW27 rotor. The virus fractions were recovered by an ISCO density-gradient fractionator with an absorbance monitor. After

concentration the virus was subjected to another cycle of sucrose-gradient centrifugation and concentration.

For antiserum production the virus was given an additional purification by equilibrium density-gradient centrifugation in Cs_2SO_4. For this purpose 1 ml of virus suspension was mixed with 2.3 ml of Cs_2SO_4 solution (0.535 kg/l), and overlaid with paraffin oil. The Cs_2SO_4 gradients were centrifuged for 19 h at 30000 rpm in a Beckman SW41 rotor. The virus was recovered by puncturing the bottom of the tubes and collecting appropriate fractions. The Cs_2SO_4 was then removed by dialyzing against PCA buffer. The virus was fixed by dialyzing the suspension overnight against 2% methanal in PCA buffer. The excess methanal was removed by extensive dialysis against PCA buffer.

Sedimentation coefficients and buoyant density. Sedimentation coefficients at infinite dilution were determined by the graphical method of Markham (1960) using a Spinco Model E ultracentrifuge with schlieren optics. The buoyant density in Cs_2SO_4 was determined after centrifuging the virus to an equilibrium as described above. Three-drop fractions were then taken from the bottom of the tubes, and 1 ml of water was added to alternate fractions which were monitored at 260 nm. Refractive indices at 25°C (n_D^{25}) were measured of the other fractions and the buoyant density at 25°C (ρ^{25}) of the virus was determined by using the relation $\rho^{25} = 12.1200n_D^{25} - 15.1662$ (Vinograd and Hearst, 1962).

Molecular masses of the nucleic acids and the coat protein. Samples were prepared for nucleic-acid and protein molecular-mass determinations by resuspending virus pellets, following high-speed centrifugation, in 0.1 M phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol. The suspensions were incubated for 3 h at 37°C and stored frozen at –20°C until required.

Nucleic-acid determinations were done in 2.6% polyacrylamide gels at 60°C using the method of Loening (1967, 1969). The nucleic acids of cowpea chlorotic mottle virus (CCMV), which have molecular masses of 1.20, 1.07, 0.81 and 0.25 $\times 10^6$ under these conditions (Verduin, 1978), were used as internal markers. Gels were prerun at $2\frac{1}{2}$ mA/gel for 30 min and run at $2\frac{1}{2}$ mA/gel for 6 h.

Protein molecular-mass determinations were done on 10% polyacrylamide gels at room temperature using the method of Weber and Osborn (1969). BDH molecular-mass marker mixture, product number 44223 2U (molecular masses 14300–71500), was used as a standard. Gels were run for 5 h at 6 mA/gel.

Electron microscopy. Crude sap preparations were made by finely chopping virus-containing fresh leaves of *C. quinoa*, or leaf pieces fixed for 90 min in 10% methanal solution, in 2% aqueous PTA, pH 6.5 or pH 3.5. Purified suspensions were directly exposed to the carbon-reinforced film for 1 to 2 min and then stained with 2% PTA, pH 6.5.

Serology. A rabbit was injected with purified virus (Sp20–9) fixed with 2% methanal. First two intravenous injections were given with a 4-day interval. Two weeks later an intramuscular injection with Freund's adjuvant followed. Because of poor titre development six weeks thereafter two more intravenous injections were given with a 5-day interval. After another six weeks two more of such injections followed with a 2-day interval. Per injection all virus purified from c. 600 g of *C. quinoa* leaves was administered.

The GE36 antiserum used was the one described by Maat and Vink (1971), originally having a titre of 64, and not reacting with material from healthy plants.

Most antisera to 37 other viruses were from our collection. The antiserum to the virus of bean rugose mosaic had earlier been obtained from F. J. Morales, Cali, Colombia; of bean southern mosaic from H. E. Waterworth, Glen Dale, USA; of beet cryptic from R. F. White, Harpenden, England; of elm mottle from A. T. Jones, Invergowrie, Scotland; of Myrobalan latent ringspot from J. Dunez, Bordeaux, France; of parsnip yellow fleck from A. F. Murant, Invergowrie, Scotland; of pear pollen from J. A. Tomlinson, Wellesbourne, England; of *Pelargonium* leaf curl from M. Hollings, Littlehampton, England; of raspberry bushy dwarf from A. F. Murant; of sowbane mosaic from G. Morvan, Montfavet, France; of one of the strains of tobacco streak from R. W. Fulton, Madison, USA; of tomato bushy stunt from M. Hollings; and of virus MF from J. C. Devergne, Antibes, France.

The serological test applied was the agar double-diffusion test and the agar concentration was 1% in saline containing 0.05% sodium azide. For all tests unfixed purified or partially purified virus was used.

Results

Host range and symptoms. The virus was isolated several times from seed of a number of spinach cultivars. Infected and virus-free plants from such seed kept under observation in the glasshouse never showed any difference. Plants of 17 spinach cultivars, developing from virus-free seed, did not visibly react to inoculation with Sp20–9, nor did plants of six of these cultivars to inoculation with GE36, as compared with un-inoculated controls.

The results of host-range tests as well as observations by Van der Meer (1968) are summarized in Table 1. Seventeen out of 36 species we have tested were susceptible to Sp20–9 and 17 species, mostly the same, were susceptible to GE36. Van der Meer (1968) listed four other susceptible hosts. All but *C. amaranticolor*, *Cucumis sativus*, *Petunia hybrida* and *Phaseolus vulgaris* 'Bataaf' were invaded systemically by Sp20–9 and in most species, except *Axyris amaranthoides*, *C. amaranticolor*, *C. quinoa* and *P. vulgaris* 'Bataaf', infection was symptomless. Our results with GE36 were in line with those by Van der Meer (1968) and no appreciable or only slight differences between Sp20–9 and GE36 were detected.

Plants of *Axyris amaranthoides* reacted with severe stunting to Sp20–9 and GE36. Symptoms were most severe with GE36 and plants with Sp20–9 recovered from disease in c. $1\frac{1}{2}$ months.

Most varieties and cultivars of *Beta vulgaris* often reacted with small chlorotic or etched rings on the inoculated leaves (Fig. 1). The chlorotic rings mostly disappeared.

Chenopodium amaranticolor produced numerous pin-point local lesions about 3 days after inoculation. These lesions were small and dry on lower leaves and slightly larger and more chlorotic on higher leaves (Fig. 2), and were identical for both virus isolates. No systemic virus infection ensued.

C. quinoa reacted to both isolates after 3 to 4 days with moderate numbers of chlorotic local lesions, which were often vague and transient and in younger inoculated leaves accompanied by a yellowish mottling (Fig. 3). Systemic symptoms usually started about 10 days, but sometimes as early as 7 days, after inoculation with slight

Fig. 1. Leaf of sugar beet 13 days after inoculation with Sp20–9.

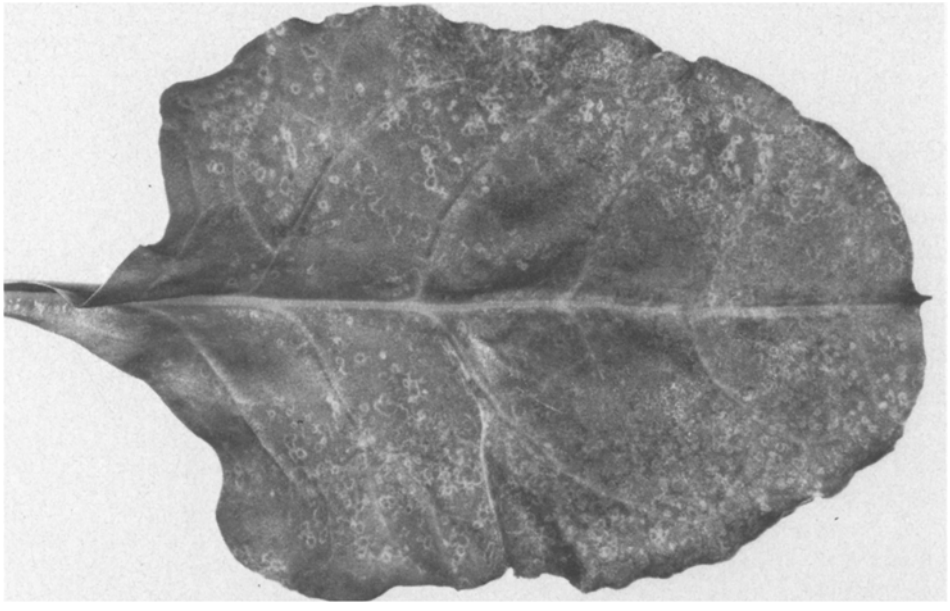

Fig. 1. Suikerbieteblad 13 dagen na inoculatie met Sp20–9.

Fig. 2. Local lesions in *Chenopodium amaranticolor* 7 days after inoculation with Sp33A.

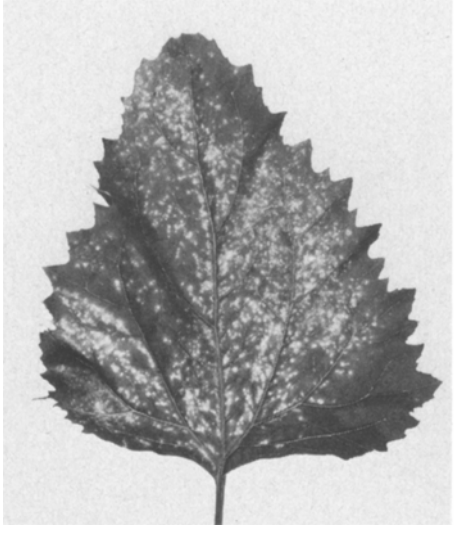

Fig. 2. Lokale lesies in Chenopodium amaranticolor 7 dagen na inoculatie met Sp33A.

Fig. 3. Local reaction in *Chenopodium quinoa* 14 days after inoculation with Sp20–9.

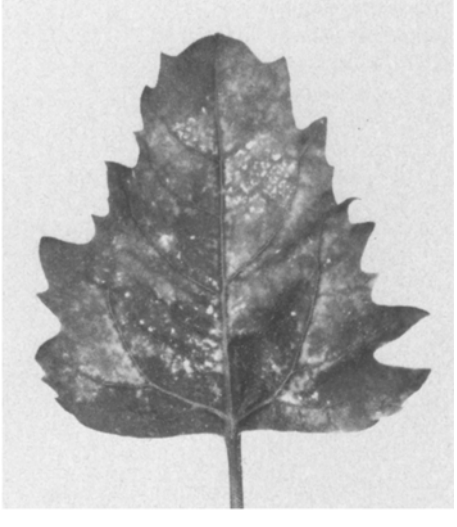

Fig. 3. Lokale reactie in Chenopodium quinoa 14 dagen na inoculatie met Sp20–9.

Table 1. Host-range tests.

Test species	Numbers of plants tested	Present research						Van der Meer (1968)	
		Sp20–9			GE36			GE36	
Amaranthus tricolor									s
Ammi majus	4	l	–		–	–			
Antirrhinum majus	3	l	–		l	s			
Apium graveolens var *dulce*	3	–			–				
var. *rapaceum*	2	–			–				
Atriplex hortensis									s
Axyris amaranthoides	4	L	S			S			S
Beta vulgaris fodder beet 'Brigadier'	5	l	s		l	s			
'Corona'	5	(L)	–		(L)	s			
'Oscar'	5	(L)	s		(L)	s			
'Polygroeningia'	5	(L)	s		l	–			
sugar beet	5	L			l	(s)			
var. *cicla* spinach beet	5	(L)	s		(L)	s			
var. *rubra* red beet	5	(L)	s		?	s			
Celosia cristata		l	s	2/2	l	s	2/2		s
C. plumosa									S
Chenopodium amaranticolor		L	–		L			L	–
Ch. quinoa		L	S		L	S		L	S
Ch. urbicum									s
Crotalaria sp.	4	–	–		–	–			
Cucumis sativus 'Gele Tros'		(L)	–						
	8 and 6	(L)	–		L	s			
Cyamopsis tetragonoloba	4	–	–		–	–			
Datura stramonium	3		–			–			
Gomphrena globosa		l	s		l	s			
Heliotropium peruvianum									s
Hyoscyamus niger	4	–	–		–	–			
Lactuca sativa 'Attractie'	3	–	–		–	–			
Lycopersicon esculentum		–	–						
Medicago sativa	4	–	–		–	–			
Nicotiana clevelandii		l	s						
N. glutinosa	8	l	s		l	s			S
N. rustica	2	l	s		l	s			s
N. tabacum 'Samsun'		l	s		l	s			
	3	l	s		l	s			
	5	l	s			s	5/5		
'White Burley'		l	s		l	s			
			s			–			
	2		s	2/2		s	2/2		
	5					s			
'Xanthi'		l	s		l	s			
some varieties									S

Table 1. Continued.

Test species	Numbers of plants tested	Present research				Van der Meer (1968)
		Sp20–9		GE36		GE36
Petroselinum crispum	3		–		–	
Petunia hybrida		–	–			
	2	l	–	l	s	
Phaseolus vulgaris 'Amanda'	16–12	–		–		
'Bataaf'	8	L		L		
'Beka'						L
'Corene'	16	–		–		
'The Prince'	8–11	–		–		
'Princesco'	2	–	–	–	–	
Phlox drummondii	3	l	s	l	s	
Physalis floridana	3	–		–		
Pisum sativum 'Koroza'		–	–			
'Rondo'		–	–			
Raphanus raphanistrum	5–4	–		–		
Spinacia oleracea 'Advance'	40	–	–			
'Dynamo'	20		s		s	
'Energie F1'	40		s			
'Ezelsoren'	40	–*	–*			
'Grand Flay'	40	–*	–*			
'Indian Summer'	40		s			
'Mazurka'	40	–*	–*			
'New Even F1'	40		s			
'Nores 20–22'	20		s		s	
'Norvak'	40		s			
'Palona F1'	40	–*	–*			
'Summic F1'	40	–*	–*			
'Vikinum'	20		s		s	
'Viroka'	20		s		s	
'Vital (R)'	20		s		s	
'Vroeg Reuzenblad'	40	–*	–*			
'Wintra'	20		s		s	
Tetragonia expansa	2	l	s	l	s	
Trifolium incarnatum	4	–	–*	–	–*	
T. pratense	4	–	–*	l	–	
T. repens	4	–	–*	–	–*	
Vicia faba 'Kompakta'		–	–			
Vigna sinensis 'Early Ramshorn'	16	–	–*	–	–*	
Vinca rosea	1	–	–			

l = latent local infection; L = local symptoms; s = latent systemic infection; S = systemic symptoms; – = no symptoms, no infection as demonstrated by back inoculation; –* = no symptoms, but infection not tested by back inoculation.

Tabel 1. Waardplantproeven.

leaf rolling followed by chlorotic vein banding, yellow mottling, growth reduction and sometimes desiccation of young leaves, with symptom severity depending on inoculum dose (Fig. 4). Usually symptoms were mildest with GE36. Infection of seedlings from the seed was mostly characterized by leaf asymmetry, slight discoloration, slight downward curling of leaf margins (Fig. 5) and often but not always by growth reduction.

Cucumis sativus reacted to both isolates in most of the plants with few more or less transient chlorotic local lesions.

All *Nicotiana* species tested contracted symptomless infection with both isolates, although less readily with GE36 in *N. rustica* and *N. tabacum* 'White Burley', and in lower concentration as shown by back inoculation.

Fig. 4. Systemic symptoms in *Chenopodium quinoa* after inoculation with Sp20–9 from *Nicotiana tabacum* 'Xanthi' with different virus content. Right, healthy control.

Fig. 4. Systemische symptomen in Chenopodium quinoa na inoculatie met Sp20–9 vanuit Nicotiana tabacum 'Xanthi' met verschillend virusgehalte. Rechts, gezonde controle.

Fig. 5. Asymmetric leaves of *Chenopodium quinoa* plants grown from seed infected by Sp20–9, 51 days after sowing. Upper left, healthy control.

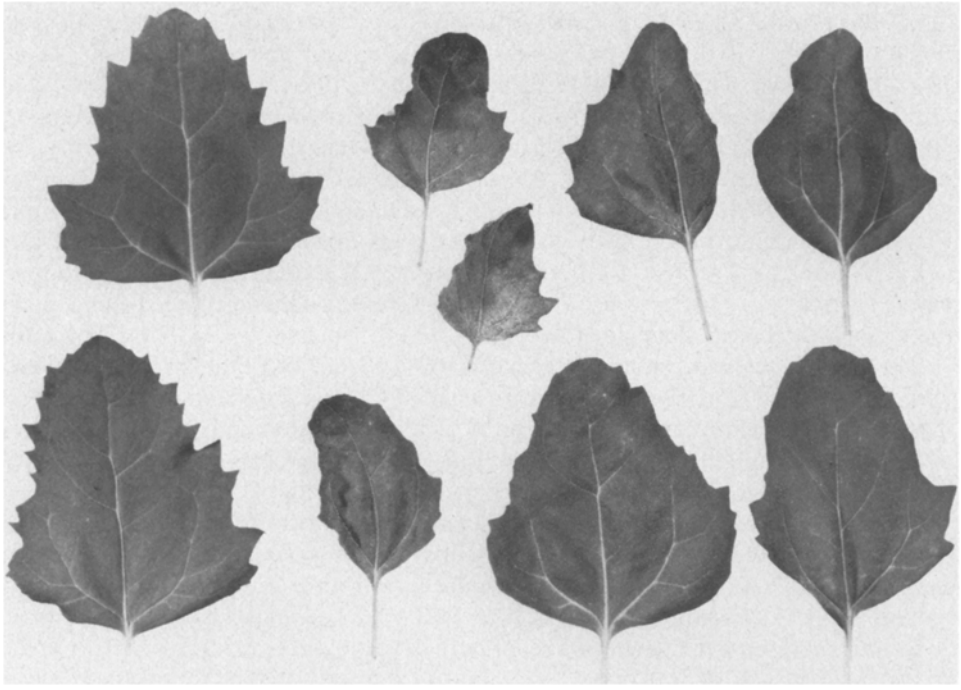

Fig. 5. Asymmetrische bladeren van planten van Chenopodium quinoa opgekweekt uit met Sp20–9 geïnfecteerd zaad, 51 dagen na uitzaai. Boven links, gezonde controle.

In *Phaseolus vulgaris* 'Bataaf' distinct necrotic local lesions were formed after 3 to 4 days. They resembled those obtained by Van der Meer (1968) in the cv. Beka (Fig. 4 of Van der Meer) which is closely related to 'Bataaf'. Maat and Vink (1971) used 'Bataaf' as a local lesion assay host for research on purification and serology.

Persistence of infectivity. Determinations were made twice with Sp20–9 and once with GE36 in sap from systemically infected plants of *C. quinoa*. Treated fractions were tested on *C. amaranticolor* and *C. quinoa*. Dilution end-point was both times between 10^3 and 10^4 for Sp20–9, and over 10^5 (with a steep decline after 10^3) for GE36. Thermal inactivation was between 55 and 80° and 60 and 65°C for Sp20–9 and 60 and 65° for GE36 (Van der Meer, 1968: 55–60°C). Ageing in vitro was between 13 and 20 days (with a steep decline in infectivity after 10 days) and between 4 and 5 days for Sp20–9, and longer than 9 days for GE36 (Van der Meer, 1968: c. 2 days).

Finely cut leaf material of infected *C. quinoa* and *N. rustica* dried over $CaCl_2$ in 1977 still contained infective Sp20–9 virus when tested after 21 months of storage.

Insect transmission test. Virus-free aphids, *Myzus persicae*, were first starved for 2 h and then allowed to probe or feed on infected leaves of *C. quinoa* for 20 min. They were then transferred to 10 virus-free *C. quinoa* plants, 10 aphids per plant. After 30 min they were

transferred to another series of 10 assay plants and killed with an aphicide the next morning. None of the 20 assay plants developed symptoms.

Seed transmission. Incidence in commercial seed lots was tested by using seedling tests. In 1977 the virus was detected in 5 out of 43 lots tested and amounted to 2, 6, 9, 20 and 28%, respectively. During 1978 fresh seed of most cultivars, infected in 1977 and obtained from different origins, was tested. No virus was then obtained in one cultivar. Of two other cultivars Dutch seed was free whereas Danish, East German and Polish origins contained the virus up to 32, 52 and 16%, respectively. All three Dutch origins of another cultivar did contain the virus. Two Dutch origins (up to 20%) and one French origin (22%) of a cultivar not previously tested were also found infected.

Later during 1978 samples from 99 seed lots were tested and 7 were found to contain virus in 2 and 44% of their seeds. The number of spinach cultivars detected to contain the virus in their seeds then rose to 12.

During these tests cucumber mosaic virus was detected four times in three cultivars and distinguished from Sp20–9 virus by its lack of systemic infection in *C. quinoa* and its subsequent reaction on cucumber, or by its direct reaction on cucumber seedlings often added as a differential host for this virus. Infection percentages did not exceed 4. Another cultivar contained some tobacco rattle virus (2 out of 50 seedlings).

When comparing *different biological assays* for seed infection, results with a sample of one cultivar were as follows: direct assay of dry seeds ground individually in water: 21/50 or 42%; assay of soaked seeds ground individually in water: 24/50 or 48%; individual assay of seedlings sown in flats: 13/25 or 52%. With another cultivar seed coats and embryos were also tested separately: embryos from dry seeds 4/10 or 40%; seed coats from dry seeds 0/10 or 0%; embryos from seeds soaked for one day 9/20 or 45%; seed coats from the same 0/20 or 0%; whole seeds soaked for one day 9/20 or 45%; whole germinated seeds 3/20 or 15%; germinated seeds, plantlets 2 cm long 4/15 or 26%; seedling test 4/50 or 20%.

Seed transmission in artificial host species was also studied. The seed tested had been harvested from plants inoculated when young. Seed testing was by visual observation of seedlings for *C. quinoa* (Fig. 5) and by individual or group testing for infectivity for all other hosts. Results are given in Table 2. In seeds of *C. quinoa* the Sp20–9 virus could also be easily detected by grinding an average of dry seeds with water and inoculation onto test plants. When 10 dry seeds were tested individually, 9 were found infected. The Sp20–9 virus could also be easily detected in all three groups of 10 seeds of infected 'Samsun' tobacco plants and in all three groups of 10 seeds of infected 'Xanthi' tobacco plants. Ten seeds of 'Samsun' tobacco tested individually only showed infection in four although in seedling tests over 70% of the seeds had been found to contain the virus.

Virus purification. Using the method described, we obtained after the two cycles of differential centrifugation a virus suspension that was still contaminated with normal plant constituents as can be seen from the UV-absorption pattern after the first sucrose-gradient centrifugation (Fig. 6A). If, however, the material from the indicated virus zone was concentrated and subjected to another sucrose-gradient centrifugation, highly purified preparations were obtained (Fig. 6B). In sucrose-gradient centrifugation the virus sedimented in two main zones. In between these a minor component could be seen, especially when gradients were loaded with relatively small amounts of virus (Fig. 6C).

Table 2. Results of seedling tests for the study of seed transmission in artificial hosts.

Host species	Sp20–9				GE36		
	number of seedlings infected over number tested			percent infection	number of seedlings infected over number tested		percent infection
	visual observation	individual testing for infectivity	average testing for infectivity		visual observation	average testing for infectivity	
Celosia cristata		8/15		53		0/70[1]	0
Chenopodium quinoa[2]	47/51[3]			92	39/89		44
	18/20			90			
	42/46			91			
Gomphrena globosa						0/18	0
Nicotiana rustica		6/20		30		2/20	10
Nicotiana tabacum 'Samsun'		18/25		72		5/20	25
'White Burley'		18/20		90			
'Xanthi'		33/35		94		0/36	0
						3/20	15
Tetragonia expansa			0/4	0		0/15	0

[1] Tested for infectivity in groups of 10 seedlings.
[2] All three samples were from the same seed lot.
[3] From 11 seedlings with doubtful to no symptoms and therefore tested individually for infectivity only 4 without any abnormality proved virus free.

Tabel 2. Resultaten van de opkweektoetsen voor het onderzoek van zaadoverdracht bij kunstmatige waardplanten.

Fig. 6. UV-absorption patterns of Sp20–9 preparations after sucrose-gradient centrifugation: A. after one centrifugation, B. after two centrifugations, C. after loading with a small amount of virus, showing three centrifugal components. Sedimentation is from left to right.

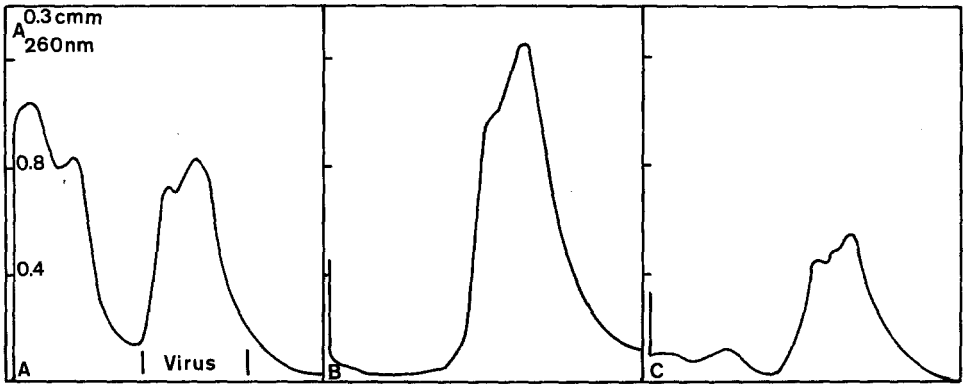

Fig. 6. UV-absorptiepatronen van preparaten van Sp20–9, na suikergradiëntcentrifugering: A. na één centrifugering, B. na twee centrifugeringen, C. na belading met een kleine hoeveelheid virus waardoor de drie centrifugale componenten zichtbaar zijn. Sedimentatie is van links naar rechts.

Sedimentation coefficients and buoyant density. In the analytical ultracentrifuge the virus sedimented in three peaks. The sedimentation coefficients at 20°C and infinite dilution in PCA buffer were 87, 98 and 108 S.

In Cs_2SO_4-gradients the virus reached equilibrium in one zone. Its buoyant density was 1.269×10^3 kg. m^{-3}.

Molecular masses of the nucleic acids and the coat protein. On 2.6% polyacrylamide gels five nucleic-acid bands were found as represented in Fig. 7A and indicated with 1, 2, 3, 4 and 5 in order of decreasing molecular mass. If gels containing the five nucleic acids were incubated for $3\frac{1}{2}$ h in 0.001% ribonuclease A (bovine pancreas type 1A) in 10 mM phosphate buffer pH 7, treated for 5 min at 100°C to destroy DNase activity, the nucleic acids were digested (Fig. 7B), indicating that Sp20–9 contains ribonucleic acids. Using the CCMV RNAs as internal markers the relative molecular masses of the RNA 1, 2, 3, 4 and 5 were calculated to be 1.30, 1.18, 0.91, 0.35 and 0.27×10^6, respectively.

On 10% gels a single polypeptide with a relative molecular mass of 28×10^3 was found.

Electrophoretic mobility of virions. Using 2.5% polyacrylamide gels and Loening's methods (1967, 1969), but PCA buffer as electrophoresis buffer, Sp20–9 separated in at least two bands (Fig. 8). The fast moving band corresponded with the slow centrifugal component, the slow one with the fast centrifugal component.

Electron microscopy. Spherical virus particles could only be detected with great difficulty in crude sap preparations from *C. quinoa* plants with clear local and systemic symptoms, and somewhat easier in preparations from leaf pieces after fixation in methanal. Results with PTA at pH 3.5 were not better than at pH 6.5. The virions in

Fig. 7. Polyacrylamide gel electrophoresis of the RNAs of Sp20–9. A. Gel incubated for $3\frac{1}{2}$ h in 10 mM phosphate buffer, pH 7. B. Gel incubated for $3\frac{1}{2}$ h in 0.001% RNase in 10 mM phosphate buffer, pH 7. The RNAs were stained with toluidin blue.

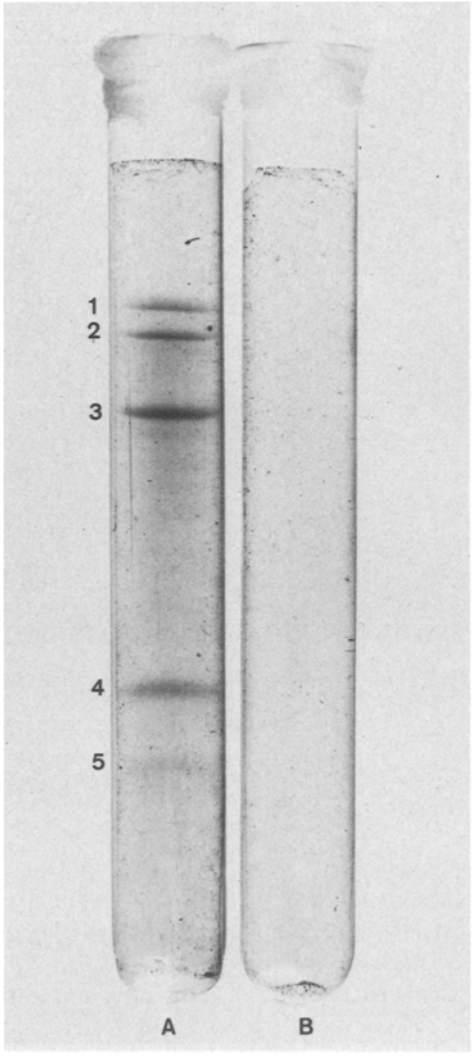

Fig. 7. Polyacrylamide-gelelektroforese van de RNA's van Sp20–9. A. Gel geïncubeerd gedurende $3\frac{1}{2}$ h in 10 mM fosfaatbuffer, pH 7. B. Gel geïncubeerd gedurende $3\frac{1}{2}$ h in 0,001% RNase in 10 mM fosfaatbuffer, pH 7. De RNA's werden gekleurd met toluidineblauw.

Fig. 8. Polyacrylamide gel electrophoresis of Sp20–9 virions. The virus zones were stained with comassie brilliant blue.

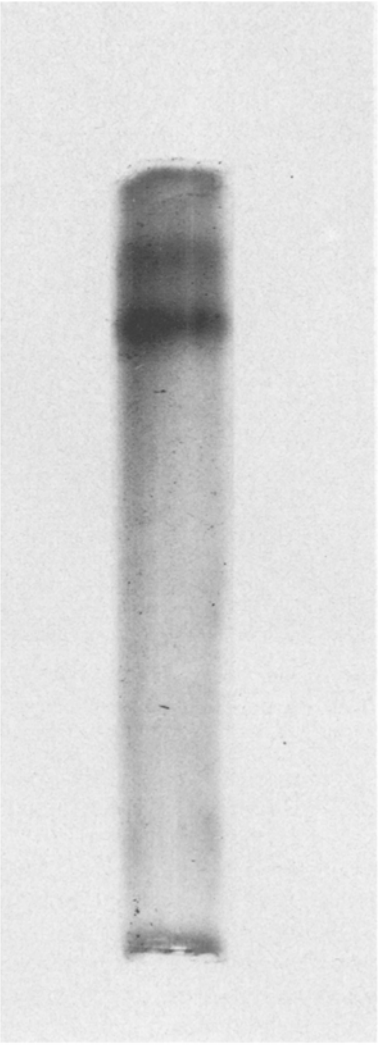

Fig. 8. Polyacrylamide-gelelektroforese van de virions van Sp20–9. De viruszones werden gekleurd met 'comassie brilliant blue'.

Fig. 9. Electron micrograph of purified Sp20–9, stained with 2% PTA in water, pH 6.5. Bar represents 100 nm.

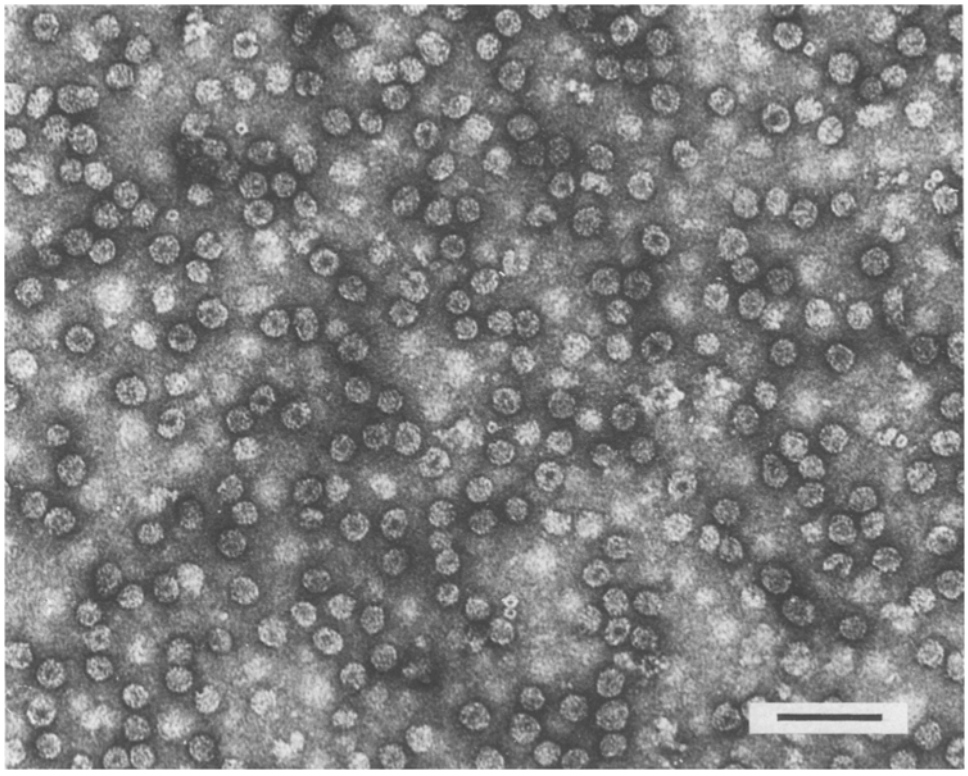

Fig. 9. Elektronenmicroscoopfoto van gezuiverd Sp20–9, gecontrasteerd met 2% PTA in water, pH 6,5. De vergrotingsstreep geeft 100 nm weer.

purified preparations were irregularly shaped and some of them were bacilliform (Fig. 9). The average diameter of the spherical particles was 27 nm.

Infectivity of purified preparations. After inoculation of test plants with a purified suspension as used for further biophysical characterization of the virus, the plants reacted as characteristic of Sp20–9: *C. amaranticolor* and *C. quinoa* reacted with local lesions and *C. quinoa* with typical systemic symptoms. In *Gomphrena globosa*, *Nicotiana glutinosa*, *N. rustica* and *N. tabacum* 'White Burley' and 'Xanthi' infection was systemic and symptomless.

For a further study of the involvement of the different components in infectivity, 0.25 ml portions of virus preparations, which were degraded with SDS and 2-mercaptoethanol, were centrifuged on 10–40% sucrose gradients in 0.1 M phosphate buffer, pH 7, containing 0.1% SDS, for 16 h at 37000 rpm in a Beckman SW41 rotor. In this way the virus could be separated into three fractions: the coat protein, RNA 1+2+3, and RNA 4+5 (Fig. 10). The latter two fractions were precipitated with ethanol and again run on sucrose gradients to improve purity. Checks on polyacrylamide gels

Fig. 10. UV-absorption pattern of degraded Sp20–9 after separation by sucrose gradient centrifugation. Sedimentation from left to right.

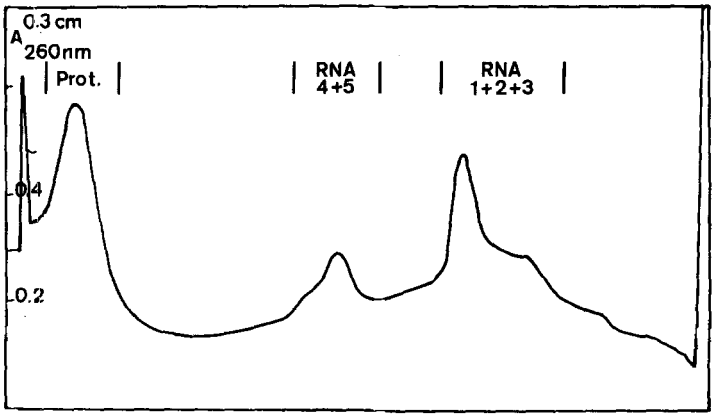

Fig. 10. UV-absorptiepatroon van afgebroken Sp20–9 na scheiding door middel van suikergradiëntcentrifugering. Sedimentatie van links naar rechts.

proved that RNA 1+2+3, RNA 4+5 and the protein were no longer cross-contaminated after these treatments. The three preparations were inoculated separately and in the combinations indicated in Table 3. The results show that RNA 1+2+3, RNA 4+5, and the protein alone are not infectious. A mixture of RNA 1+2+3 and RNA 4+5 is infectious, as is the mixture of RNA 1+2+3 and the protein. The virus progenies of inocula 3, 4 and 5 in Table 3 were purified and analyzed on sucrose gradients. After degradation the nucleic acids and the proteins were also analyzed on polyacrylamide gels. The progenies appeared to be completely identical to complete Sp20–9. So RNA 4+5 can be replaced by the coat protein, and the three large RNAs contain the complete genetic information.

GE36 was purified and analyzed by the same method as just described. It showed

Table 3. Infectivity of the RNA components of Sp20–9 and the effect of its coat protein on infection. Each inoculum was tested on two *C. quinoa* plants. + : symptoms on test plant. – : no symptoms.

Inoculum	Symptom development
1. Prot.	– –
2. RNA (1+2+3)	– –
3. RNA (1+2+3) + RNA (4+5)	+ +
4. RNA (1+2+3) + prot.	+ +
5. RNA (1+2+3) + RNA (4+5) + prot.	+ +
6. RNA (4+5)	– –
7. RNA (4+5) + prot.	– –

Tabel 3. Infectiositeit van de RNA-componenten van Sp20–9 en het effect van het manteleiwit op infectie. Elk inoculum werd getoetst op twee planten van C. quinoa. + : symptomen op toetsplant. – : geen symptomen.

exactly the same multicomponent behaviour in sucrose-gradient centrifugation and it reached equilibrium in Cs_2SO_4 at the same density as Sp20–9 did. It also had 5 RNAs and one coat protein, which had the same relative molecular masses as those of Sp20–9, because they comigrated in polyacrylamide gel electrophoresis.

Serology. Fourteen days after the third, fifth and seventh injections with Sp20–9 the homologous titres of antiserum were 4, 32, and 32, respectively. In later tests the second bleeding had a titer of 64. In NaCl-agar both virus isolates Sp20–9 and GE36 reacted with two distinct lines (Fig. 11). When both antigens alternated in wells surrounding a common central well with any of the two antisera the two lines just-mentioned fused without spur formation. Both antisera reacted up to the same dilution with homologous and heterologous antigen (32 for GE36 antiserum and 64 for Sp20–9 antiserum). The most rapidly diffusing component reacted up to lower dilutions (8 for GE36 antiserum and 16 for Sp20–9 antiserum and up to the same dilution for both antigens). When at the end of the experiments agar in 0.05 M phosphate-citric acid pH 7 was used instead of NaCl-agar, only a single line was formed. Neither antiserum reacted with crude sap or concentrated preparations from healthy plants.

No reactions were obtained with purified Sp20–9 virus and antisera to alfalfa mosaic virus and any of the following 36 spherical viruses: viz. of apple mosaic, *Arabis* mosaic, bean pod mottle, bean rugose mosaic, bean southern mosaic, beet cryptic, broad bean wilt, carnation mottle, carnation ringspot, cherry leafroll, cocksfoot mild mosaic, cowpea mosaic (yellow and severe), cucumber mosaic (two isolates), elm mottle, lilac ring mottle, myrobalan latent ringspot, parsley latent, parsnip yellow fleck, pear pollen, *Pelargonium* leaf curl, *Pelargonium* L128, *Prunus* necrotic ringspot, raspberry bushy dwarf, raspberry ringspot, red clover mottle, sowbane mosaic, strawberry latent ringspot, tobacco necrosis (two strains), tobacco ringspot, tobacco streak (two strains),

Fig. 11. Reactions of antiserum to GE36 (A) with GE36 (1) and Sp20–9 (2).

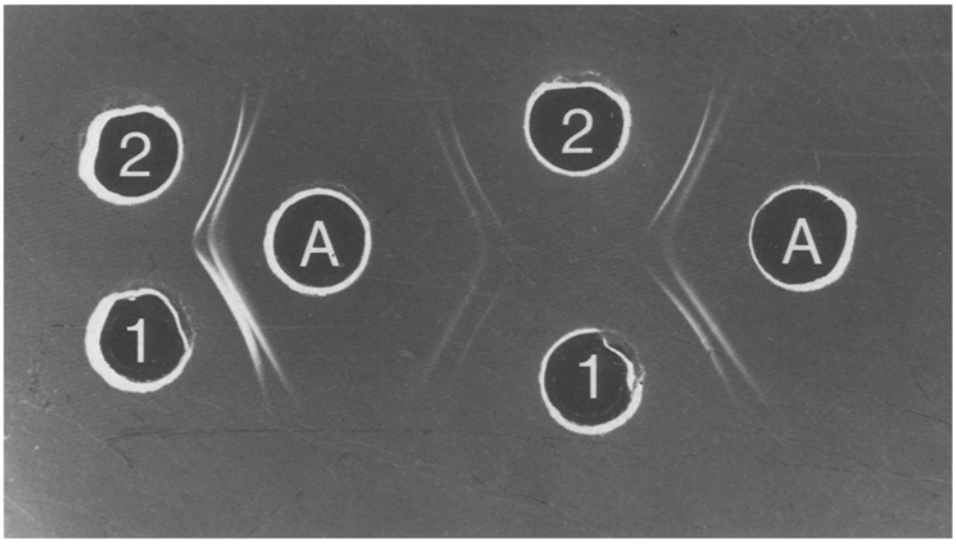

Fig. 11. Reacties van het GE36-antiserum (A) met GE36 (1) en Sp20–9 (2).

tomato aspermy (chrysanthemum isolate), tomato black ring, tomato bushy stunt, tomato ringspot, turnip yellow mosaic, and virus MF.

In a tentative experiment GE36 antiserum was used to test crude sap of *C. quinoa* with Sp20–9 and two other isolates of the virus, and results were positive.

Discussion

This paper presents a detailed description of the spinach isolate Sp20–9 and a further characterization of the GE36 virus, originally isolated by Van der Meer (1968) and studied in some detail by Maat and Vink (1971). Infectivity tests with purified and serologically and biophysically characterized Sp20–9 have confirmed this virus to be the virus repeatedly isolated from spinach.

We have not detected any appreciable difference (biologically, serologically nor biophysically) between Sp20–9 and GE36 virus. The latter tended to be less virulent than Sp20–9, and to be less easily seed-transmitted. Van der Meer (personal communication) found slight differences in virulence between different isolates from fruit trees. Symptom severity also depended on conditions (see also Van der Meer, 1968, for GE36) and on inoculum dose (Fig. 4). We did not obtain a mild mosaic in *N. glutinosa* as mentioned by Van der Meer (1968).

Hence, our spinach virus is identical with the GE36 virus, the name of which theoretically should have priority. Although described as a virus from apple and pear, the GE36 virus has neither been detected directly in apple and pear nor in *C. quinoa* inoculated from apple and pear, but only via other hosts inoculated from these fruit trees and by back inoculation from those hosts onto *C. quinoa* (Van der Meer, 1968). The virus could not be recovered from apple seedlings grafted with infected *C. quinoa*, although apple stem grooving (Van der Meer, 1976) and chlorotic leaf spot virus, introduced in this manner, could, but it could be recovered from the herbaceous rosaceous species *Sanguisorba minor* (Van der Meer, 1972). So its real origin could not be proved. Van der Meer (1973) detected 25–30% seed transmission of GE36 in *C. quinoa* and suggested that the virus had originated from symptomless plants of *Chenopodium* species used. However, he could not detect it when testing seed samples of a number of such species (Van der Meer, personal communication). The seed transmission we have now detected in a series of test plants further supports the theory of its origin from test species. So far, spinach is the only proven natural host. We therefore now feel free to describe it as a spinach virus in view of its high natural incidence in spinach seed. We have named it *spinach latent virus* (SpLV) since we have never observed symptoms in spinach plants grown from infected seed, nor been able to induce symptoms in any of 17 spinach cultivars inoculated. The name does not conflict with the one tentatively used by Štefanac (1978) for a most probably identical virus isolated in Yugoslavia.

GE36 had earlier been found not to be serologically related to any of 22 different spherical viruses and alfalfa mosaic virus (Maat and Vink, 1971). Like GE36, the Sp20–9 is weakly immunogenic. With Sp20–9 we have now shown that SpLV is not related to any of 36 spherical plant viruses including seven nepoviruses, in contrast to what could have been inferred from the high rates of seed transmission. Although SpLV has five instead of four RNAs, all other properties determined i.e. the relative molecular masses of its RNA species, the single zone in equilibrium Cs_2SO_4 gradients, its multicomponent behaviour in polyacrylamide gels and sucrose-density gradients,

meet criteria of Lister et al. (1972) for inclusion of SpLV in the ilarvirus group. Its tripartite genome and its coat protein dependency for infectivity also indicate that SpLV is a member of that group (Gonsalves and Fulton, 1977). The shape of the virions of SpLV, which is very irregular, and the occurrence of a small number of bacilliform particles, can be considered as further arguments to classify the virus in that group. Oval or bacilliform particles have been found for members and possible members of the ilarvirus group, i.e. alfalfa mosaic (Gibbs et al., 1963), black raspberry latent (Converse and Lister, 1969), citrus leaf rugose and citrus variegation (Gonsalves and Garnsey, 1976), elm mottle (Jones and Mayo, 1973), lilac ring mottle (Van der Meer et al., 1976), prune dwarf (Halk and Fulton, 1978), *Prunus* necrotic ringspot (Lister and Saksena, 1976), rose mosaic (Basit and Francki, 1972), and Tulare apple mosaic (Fulton, 1967). In our tests the SpLV did not react with antisera to the ilarviruses of apple mosaic, elm mottle, lilac ring mottle, *Prunus* necrotic ringspot and tobacco streak, but ilarviruses in general are poorly immunogenic and there are only some serological relationships between members within subgroups. Furthermore, several ilarviruses are seed transmitted.

Rates of seed transmission of SpLV have been found to be high, up to c. 50% in naturally infected spinach, and even higher in three of the four artificial host species in which seed transmission was detected (Table 2). Rates of transmission of isolate GE36 were considerably lower than of Sp20–9. Seed transmission makes the virus of potential importance, although symptomless in the spinach cultivars tested. Severe symptoms in *C. quinoa* and *Axyris amaranthoides* indicate that the virus might damage certain crops, provided a vector is available for spread. However, so far the only known way of natural transmission of ilarviruses is through pollen. Pollen transmission of SpLV has already been reported by Štefanac (1978). Seed infection is via the embryo, suggesting that virus may persist in seeds as long as they remain viable. Virus detectability in dry seeds, as demonstrated in our experiments, may greatly facilitate seed testing for the virus, but may be difficult for seeds of extremely small size as of *Nicotiana* species. In one instance more dry seeds were infected than were seedlings grown from such seed. Further research is required on field incidence and natural transmission.

Acknowledgments

We are greatly indebted to Miss M. P. Schor, Ing. Tan Tian Nio and Ing. W. H. M. Mosch for technical assistance.

Samenvatting

Latent spinazievirus, een nieuw ilarvirus dat bij spinazie met zaad overgaat

In 1977 kon uit 5 van de 43 en in 1978 uit 7 van de 99 getoetste partijen spinaziezaad afkomstig uit een aantal landen een klaarblijkelijk nieuw virus worden geïsoleerd zonder dat in de uit zulk zaad opgekweekte planten afwijkingen konden worden geconstateerd. Eén zaadherkomst was voor meer dan 50% geïnfecteerd. Het virus werd aangetoond in zaad van in totaal 12 verschillende spinazierassen. Tevens werd in enkele zaadmonsters komkommermozaïekvirus en in één het tabaksratelvirus

aangetroffen.

Biologisch, biofysisch en serologisch onderzoek heeft aangetoond dat het virus identiek is aan een eerder onder de code-naam GE36 beschreven virus, dat naar eerst werd vermoed, via toetsplanten was geïsoleerd uit appel en peer. Dit virus is nu vooral aan de hand van het spinazie-isolaat Sp20–9 uitvoerig gekarakteriseerd en beschreven als het *latente spinazievirus* ('spinach latent virus'). Zeventien van de 36 getoetste plantesoorten bleken vatbaar, de meeste echter symptoomloos. In *Chenopodium amaranticolor* ontstonden karakteristieke, voornamelijk droge puntlesies en op de primaire bladeren van *Phaseolus vulgaris* 'Bataaf' opvallende necrotische lokale lesies, terwijl in *C. quinoa* de systemische reactie meer opviel dan de lokale. Ook bieteblad reageerde soms met lokale symptomen.

De houdbaarheid van het infectievermogen in uitgeperst plantesap was bij verdunning 10^3–10^4, bij verhitting 60–65 °C, en bij bewaring 4–5 dagen en éénmaal zelfs langer dan 13 dagen.

Met *Myzus persicae* kon het virus niet op non-persistente wijze worden overgebracht. Zaadoverdracht werd ook aangetoond bij vier van de zes hierop onderzochte kunstmatige waardplanten. Bij *C. quinoa* en *N. tabacum* 'White Burley' en 'Xanthi' bedroeg dit percentage zaadoverdracht zelfs meer dan 90. GE36 ging minder gemakkelijk over met zaad.

Zuivering door butanol-klaring, differentiële en dichtheidsgradiëntcentrifugering leverde aanvankelijk nog met celbestanddelen verontreinigd virus op. Afzondering van de viruszone, concentrering en verdere dichtheidsgradiëntcentrifugering verschafte echter zeer zuiver virus. Dit virus sedimenteerde in een suikergradiënt in twee zones en bij lage concentratie in drie zones. De drie componenten hadden sedimentatiecoëfficienten van 87, 98 en 108 S. De zweefdichtheid van het virus in een cesiumsulfaatgradiënt bedroeg $1{,}269 \times 10^3$ kg. m^{-3}.

Bij polyacrylamidegel-elektroforese ontstonden vijf nucleïnezuurbanden, elk bestaand uit RNA met een relatieve moleculaire massa van respectievelijk 1,30, 1,18, 0,91, 0,35 en $0{,}27 \times 10^6$. De moleculaire massa van het eiwit bedroeg 28000. Intacte deeltjes verplaatsten zich in 2,5% polyacrylamide-gel als twee banden.

Met de elektronenmicroscoop konden virusdeeltjes met moeite in ruw sap worden waargenomen, wel echter iets beter na fixatie en gemakkelijk in gezuiverde preparaten. De deeltjes waren onregelmatig van vorm, c. 27 nm in diameter, en soms bacilvormig.

Bij infectieproeven met de verschillende groepen RNA-componenten en viruseiwit verkregen na SDS-afbraak bleek dat voor infectie de componenten 1, 2 en 3 nodig zijn tezamen met de componenten 4 en 5 òf het eiwit.

Het virus bleek slechts zwak immunogeen (antiserumtiter 64) en in agargel vormden zich tenminste twee lijnen, indien de agar was bereid in een fysiologische zoutoplossing, doch slechts één lijn indien de agar was bereid in 0,05 M fosfaat-citroenzuurbuffer pH 7. Het bleek serologisch identiek aan GE36 en was niet verwant aan 36 verschillende bolvormige plantevirussen en aan luzernemozaïekvirus.

De relatieve moleculaire massa's van de RNA-componenten, de uniforme zweefdichtheid in cesiumsulfaat, de aantoonbaarheid van meer dan één component in polyacrylamide-gel en bij suikergradiëntcentrifugering, het tripartite genoom en de eiwitafhankelijkheid ervan voor infectie, alsmede de onregelmatige deeltjesvorm en de kleine aantallen bacilvormige deeltjes rechtvaardigen plaatsing van het virus in de ilarvirusgroep.

References

Basit, A. A. & Francki, R. I. B., 1972. Rose mosaic virus. Bienn. Rep. Waite agric. Res. Inst. SO. Australia for 1970–71: 80.

Converse, R. H. & Lister, R. M., 1969. The occurrence and some properties of black raspberry latent virus. Phytopathology 59: 325–333.

Fulton, R. W., 1967. Purification and some properties of tobacco streak and Tulare apple mosaic viruses. Virology 32: 153–162.

Gibbs, A. J., Nixon, H. L. & Woods, R. D., 1963. Properties of purified preparations of lucerne mosaic virus. Virology 19: 441–449.

Gonsalves, D. & Fulton, R. W., 1977. Activation of *Prunus* necrotic ringspot virus and rose mosaic virus by RNA 4 components of some ilarviruses. Virology 81: 398–407.

Gonsalves, D. & Garnsey, S. M., 1976. Association of particle size with sedimentation velocity of the nucleoprotein components of citrus variegation and citrus leaf rugose viruses. Proc. 7th Conf. int. Org. Citrus Virologists., Univ. California, Riverside: 109–115.

Halk, E. L. & Fulton, R. W., 1978. Stabilization and particle morphology of prune dwarf virus. Virology 91: 434–443.

Jones, A. T. & Mayo, M. A., 1973. Purification and properties of elm mottle virus. Ann. appl. Biol. 75: 347–357.

Lister, R. M., Ghabrial, S. A. & Saksena, K. N., 1972. Evidence that particle size heterogeneity is the cause of centrifugal heterogeneity in tobacco streak virus. Virology 49: 290–299.

Lister, R. M. & Saksena, K. N., 1976. Some properties of Tulare apple mosaic and ILAR viruses suggesting grouping with tobacco streak virus. Virology 70: 440–450.

Loening, U. E., 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 102: 251–257.

Loening, U. E., 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 113: 131–138.

Maat, D. Z. & Vink, J., 1971. Purification and serology of GE36 virus from apple and pear. Neth. J. Pl. Path. 77: 73–82.

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

Meer, F. A. van der, 1968. Sap-transmissible viruses of apple and pear. Tag-Ber. dt. Akad. LandwWiss. Berl. 97: 27–34.

Meer, F. A. van der, 1972. GE36 virus. Jaarversl. Inst. Plantenziektenk. Onderz.: 74–75.

Meer, F. A. van der, 1973. Virusziekten bij groot fruit. Jaarversl. Inst. Plantenziektenk. Onderz.: 52–64.

Meer, F. A. van der, 1976. Observations on apple stem grooving virus. Acta Hortic. 67: 293–304.

Meer, F. A. van der, Huttinga, H. & Maat, D. Z., 1976. Lilac ring mottle virus: isolation from lilac, some properties, and relation to lilac ringspot disease. Neth. J. Pl. Path. 82: 67–80.

Štefanac, Z., 1978. Investigation of viruses and virus diseases of spinach in Croatia. Acta bot. croat. 37: 39–46.

Verduin, B. J. M., 1978. Degradation of cowpea chlorotic mottle virus ribonucleic acid *in situ*. J. gen. Virol. 39: 131–147.

Vinograd, J. & Hearst, J. E., 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. Progr. Chem. org. nat. Prod. 20: 372–422.

Weber, K. & Osborne, M., 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. biol. Chem. 244: 4406–4412.

Address

Instituut voor Plantenziektenkundig Onderzoek (IPO), Postbus 42, 6700 AA Wageningen, the Netherlands.