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Direct electron microscopy and serology with plant viruses in leaf material dried and stored over calciumchloride

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Abstract

Most of 43 viruses could easily be detected directly in 53 out of 66 leaf samples dried and stored over $CaCl_2$ for varying periods of time up to $20^1/_2$ years. Detection usually was with PTA pH 6.5, but alfalfa mosaic, cucumber mosaic and tomato aspermy viruses required PTA pH 3.0 to 4.0. Bean common mosaic, cowpea aphid-borne mosaic and cowpea mosaic viruses were also easily observed in newly dehydrated samples obtained for diagnosis from Morocco and Tanzania.

Broad bean wilt virus, cowpea mosaic virus and cucumber mosaic virus were detected with agar gel-diffusion tests in dry leaf material ground in buffer. This serological assay demonstrated a high concentration of cucumber mosaic virus in leaf material dried over $CaCl_2$ 20 years ago. This paper further coroborates the value of the $CaCl_2$ method of dehydration and storage of plant viruses in leaf material.

Introduction

Previous studies by McKinney (1953), Bos (1969, 1977), Barradas and Silberschmidt (1973), and others have shown that several widely differing plant viruses remain infective for extended periods of time in leaf material dried and stored over $CaCl_2$ at 4°C.

Preliminary tests with such material (unpublished) also showed that dry material could be directly assayed for viruses with the electron microscope. Independently, Ragetli et al. (1973) showed that direct inoculations with powdered dry material from leaves diseased with ten different viruses resulted in high infection rates. The inoculum was prepared by grinding the leaves in liquid nitrogen and freeze drying the finely divided tissue. As a side line they examined such dry leaf powders with the electron microscope and readily detected the virions.

There are also some reports on serological detectability of viruses in artificially dehydrated plant material, as of barley stripe mosaic and brome mosaic viruses by Scott (1961), of citrus crinkly-leaf-type virus by Garnsey and Purcifull (1969) and of potato Y and tobacco etch viruses by Purcifull and Gooding (1970). Purcifull et al. (1975) made a detailed study of the preservation for up to one year of antigenicity in crude plant extracts with thirteen plant viruses, and of their serological detection.

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Fig. 1. Electron micrographs of elongate plant viruses in leaf material dried and stored over $CaCl_2$: A. lettuce mosaic virus after 3 years of storage; B. cowpea aphid-borne mosaic virus in dry material sent in from Tanzania; C. bean common mosaic virus in dry material sent in from Morocco; D. red clover vein-mosaic virus, D^1 strain RK31 after 3 months of storage, D^{11} strain E207 after 9 years of storage; E. pea streak virus after $5^1/_2$ years of storage: F. bean yellow mosaic virus, pea necrosis strain E221, after 4 years of storage; G. onion yellow dwarf virus, after $3^1/_2$ years of storage. All preparations stained in PTA pH 6.5. Magnification bars represent 500 nm.

Fig. 1. Elektronenmicroscopische foto's van langwerpige plantevirussen in boven $CaCl_2$ gedroogd en bewaard bladmateriaal: A. slamozaïekvirus na 3 jaar bewaring; B. 'cowpea aphid-borne mosaic virus' in gedroogd materiaal ontvangen uit Tanzania; C. bonerolmozaïekvirus in gedroogd materiaal ontvangen uit Marokko; D. nerfmozaïekvirus van rode klaver, D^1 stam RK31 na 3 maanden bewaring, D^{11} stam E207 na 9 jaar bewaring; E. erwtestrepenvirus na $5^1/_2$ jaar bewaring; F. bonescherpmozaïekvirus, erwtenecrosestam E221, na 4 jaar bewaring; G. uiegeelstreepvirus na $3^1/_2$ jaar bewaring. Alle preparaten gecontrasteerd met PTA pH 6,5. Vergrotingsstaven geven 500 nm weer.

We have now tested with the electron microscope a wide range of viruses in dry leaf material from the senior author's collection and in some dry samples obtained from others for diagnosis. Some of the material was also used for direct serological testing.

Material and methods

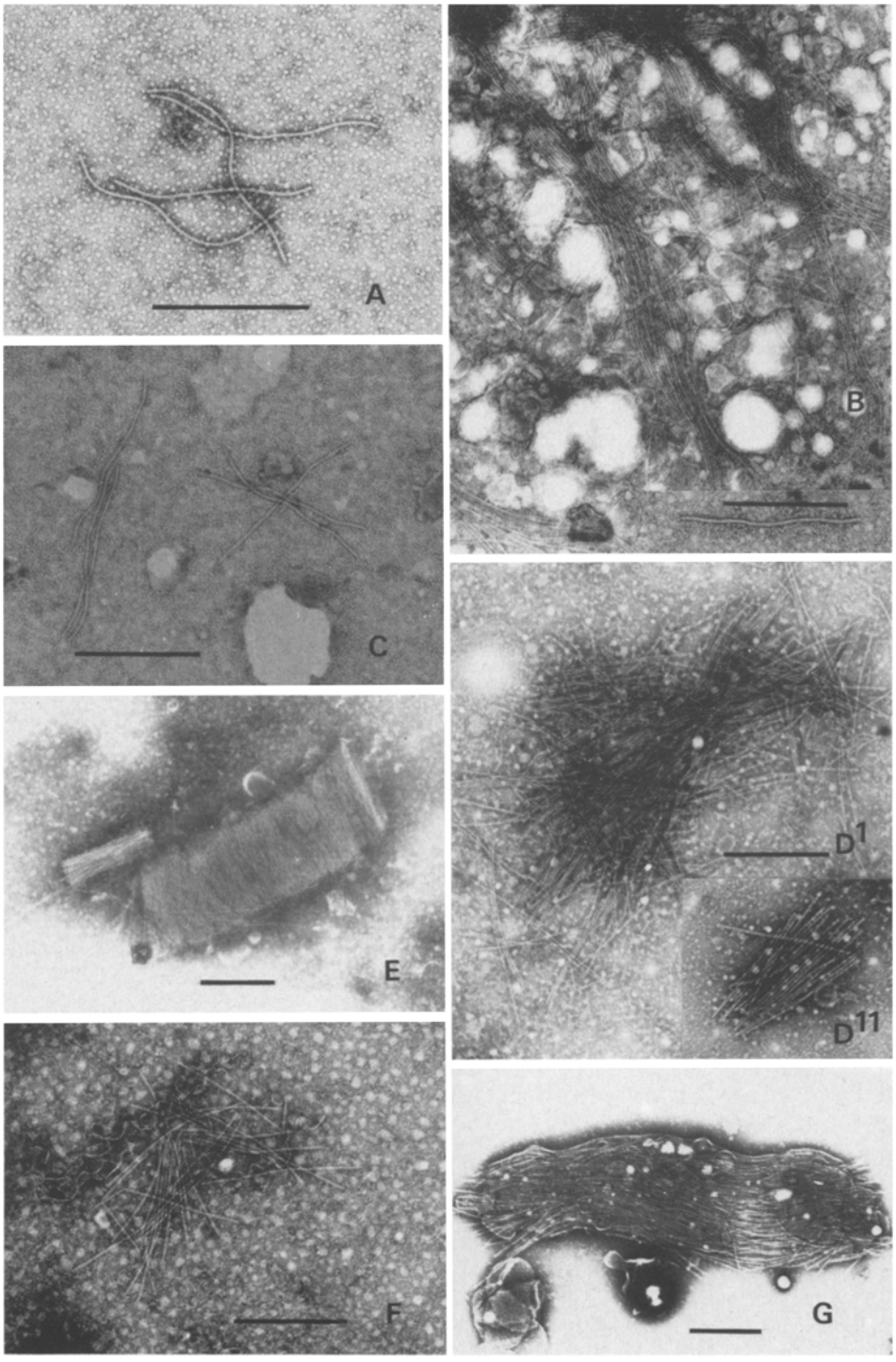
Most of the virus-containing leaf samples tested were from the senior author's collection and prepared and stored over $CaCl_2$ as described before (Bos, 1969, 1977). A dry sample with bean common mosaic virus was obtained from Dr H. Fischer, Rabat, Morocco, and some other samples with cowpea (yellow) mosaic virus and cowpea aphid-borne mosaic virus were from Dr P. N. Patel, IITA/USAID/Tanzania Project, Dar es Salaam, Tanzania.

For electron microscopy most preparations were in 2% phosphotungstic acid (PTA), pH 6.5. Small amounts of dry leaf material were chopped with a razor blade in some drops of stain on a glass microscope slide. A drop of resulting greenish liquid was then transferred with a glass capillary to a carbon-reinforced formvar-coated grid and removed after 30 seconds.

Some of the preparations were also made by grinding dry leaf samples in a mixture of three drops of 2.5% methylamine tungstate (MAT) in double-distilled water and one drop of 0.2% bacitracin and then transferring a drop to the grid where it was left for ca. 20 sec (according to Mr M. J. W. Webb, Wellesbourne, Warwick, UK, unpublished).

Viruses that were not detected with the above methods and cucumber mosaic virus and tobacco mosaic virus were tested with PTA at low pH (3 and 4) according to Milne and Masenga (1978). The dry material was ground in a few drops of PTA solution. A small drop of liquid was then transferred to the grid, left there for ca. 1 min and thereafter rinsed with five to ten consecutive drops of PTA from a pipette, drained and allowed to dry.

For serology the dry leaf material was ground with pestle and mortar in tris citric acid buffer 0.1 M pH 8.0 (1 to 10 and later 1 to 16 to imitate fresh sap on the assumption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B
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D11
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Table 1. Results[1] of direct assay for virus in dry samples of plant material from the senior author's virus collection.

Virus	Code	Date of storage	PTA at pH			MAT	Infectivity test
			3.0	4.0	6.5		
Alfalfa mosaic	425 Wisc.	1969- 4-17		++		−	+++
Arabis mosaic	Delph 1	1975- 9- 5			+	−	+
	Lig 4	1971- 9- 1			+		
Bean common mosaic	B 53	1975-12-23			+	+	
	B 54	1976- 7- 5			−		+
	F1	1970-12-24			+	+	
Bean rugose mosaic	B 116	1976-11-30			++		
Bean southern mosaic		1971- 1-14			++		
Bean yellow mosaic	B 25	1975-12-23			++		
pea mosaic str.	E 198	1975-12-16			+		
pea necrosis str.	E 221	1973-12- 6			++		
Beet mosaic	SVP	1969- 2-18			−	−	−
Broad bean wilt	E 229	1973- 9-28			−		++
Cauliflower mosaic	Brass 22	1977- 1-15			+		
Celery latent	Se 3	1971-12- 6				−	+
Clover yellow vein	E 178	1973- 9-10			++		
	E 242	1975- 5- 7			+		
Cowpea mosaic severe str.	VS	1963-10- 1			−	+	
yellow str.	YM	1963- 5-10			++	++	
Cucumber green mottle mosaic	K 3	1973- 6- 1			++		
Cucumber mosaic	B 32	1977- 8-16		+			++
	E 31	1958- 9-23		++			
	L 14	1959- 4-17		?			−
	UK 2	1959- 4-17	+				+++
	UK 6	1959- 4-17	+				
	vdW	1959- 4-17		+			
	ZB 2	1959- 4-17		+			+
Heracleum latent	Se 115	1977-11- 3			−		
Leek yellow stripe	All 11	1971-12- 3			++		
Lettuce mosaic	LS 1	1974- 3- 5			+		
Narcissus mosaic	Lisse	1968- 7-24			−		−
Odontoglossum ringspot		1969- 8-18			+++		
Onion yellow dwarf	All 41	1975- 7-21			+++		

	All 41	1975- 8-19			++			
Passiflora latent	Pass 1	1969-10-21			−		−	±
Pea early browning	VVV	1975- 6-20			++		++	
Pea enation mosaic	EnMV	1963- 6-11			++			
	E 154	1963- 2-18			++		−	+
Pea seed-borne mosaic	E 246	1974- 9-26			+		−	
Pea streak	WPSV	1972- 3-27			++			
Raspberry ringspot	vdM	1971- 7- 9			+			
Red clover mottle	Eng	1962- 7-30			−			
	Ned	1962- 4-17			+			
Red clover vein mosaic	RK 31	1977- 8- 5			++			
latent strain	E 207	1968-11-27			++			
Rice yellow mottle	Otonglo	1973- 3-18			++			
		1974- 3-28			++			
Shallot latent	All 3	1974- 6- 4			±		±	
Strawberry latent ringspot	Pe 4	1976- 3- 1					−	+
Tobacco mosaic	HR	1965- 6- 1	+++					
Tobacco necrosis	B 4	1959-11- 2			?		−	++
	Coc 2	1967- 7- 4			++			
	Fult	1966- 2-22			+++			
Tobacco rattle	Se 12 B	1972- 3-30			+		+	
Tobacco streak	Sb 3	1970- 3- 2			−			
Tomato aspermy	Sm 1.12.1	1965- 3-19	+	+	−		+	++
Tomato black ring	vH Tu	1971- 7-28			+			
Tomato mosaic	LS 121431	1969- 3- 3			+			
Turnip mosaic	Brass 2	1972-11- 8			+			
	Brass 17	1977- 1- 5			+			
	Brass 25	1977- 1- 5			++			
Turnip yellow mosaic		1963- 8-26			+			
Watermelon mosaic	K 2	1971- 9- 3			+			
White clover mosaic	vdW	1975- 2-26			++			
Wisteria vein mosaic	Wis 2B	1966- 2-28			+			
	Wis 33	1977- 8- 5			+			

[1] The number of + signs indicates concentration of virus particles in electron microscope preparations or number of local lesions on test plants; ± very few particles, ? presence of particles questionable, − no particles or no infectivity.

Tabel 1. Resultaten van rechtstreekse toetsing op virus in droge monsters plantemateriaal uit viruscollectie van de eerste auteur.

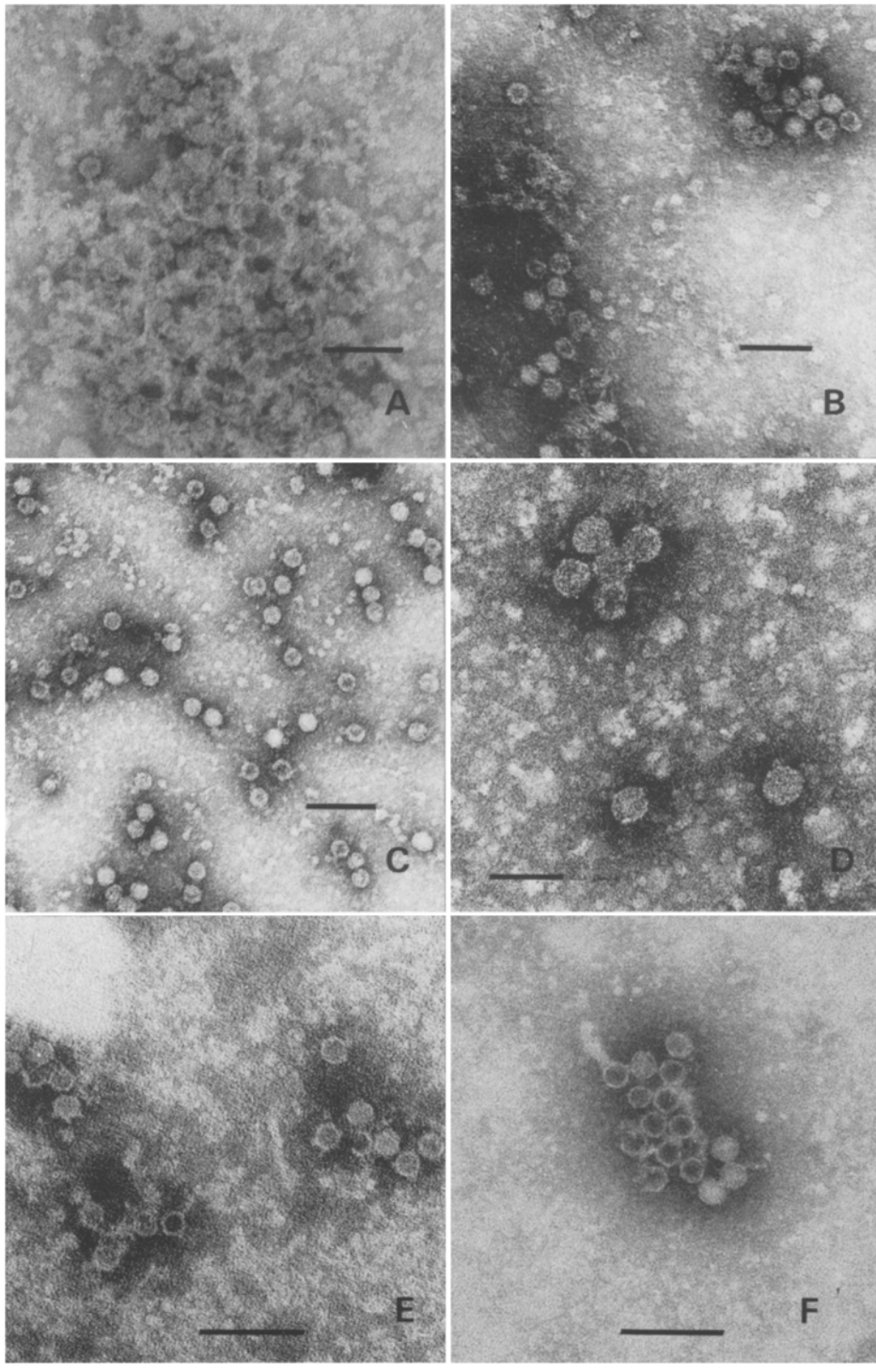
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Fig. 2. Electron micrographs of spherical plant viruses in leaf material dried and stored over $CaCl_2$: A. tomato aspermy virus after $12^1/_2$ years of storage; B. bean rugose mosaic virus after $1^1/_2$ months of storage; C. bean southern mosaic virus after 6 years of storage; D. cauliflower mosaic virus after $1^1/_2$ months of storage; E. cowpea mosaic virus yellow strain in dry material sent in from Tanzania; F. rice yellow mottle virus after $5^1/_2$ years of storage. Preparation A was in methylamine tungstate, all others in PTA pH 6.5. Magnification bars represent 100 nm.

Fig. 2. Elektronenmicroscopische foto's van bolvormige plantevirussen in boven $CaCl_2$ gedroogd en bewaard bladmateriaal: A. tomate-aspermievirus na $12^1/_2$ jaar bewaring; B. 'bean rugose mosaic virus' na $1^1/_2$ maand bewaring; C. 'bean southern mosaic virus' na 6 jaar bewaring; D. bloemkoolmozaïekvirus na $1^1/_2$ maand bewaring; E. 'cowpea mosaic virus, yellow strain' in gedroogd materiaal ontvangen uit Tanzania; F. 'rice yellow mottle virus' na $5^1/_2$ jaar bewaring. Preparaat A was in methylaminewolframaat, alle andere in PTA pH 6.5. Vergrotingsstaven geven 100 nm weer.

of ca. 6% dry matter content of fresh leaves). The moist pulp was then taken up in a small piece of cotton wool and squeezed with a handpress. The resulting liquid was used undiluted and diluted 1 to 4. Final testing was by agar gel double diffusion (Ouchterlony). In two experiments dry material with two isolates of cucumber mosaic virus (vdW 1959-4-17 and UK2 1959-4-17) was directly compared with fresh material from *Chenopodium quinoa* (inoculated leaves) and from cucumber seedlings (inoculated cotyledons and systemically infected foliage leaves separately) six and eight days after direct inoculation with the same isolates from the dry collection.

Most of the dry leaf samples in which no particles could be detected with the electron microscope were also tested for infectivity by grinding some dry material in water and direct inoculation onto appropriate test plants (see Bos, 1969).

Results

Electron microscopical assay of stored dry plant material yielded results as summarized in Table 1. Some of the results are represented in Figs 1 and 2. Fifty-five samples out of 66 with different isolates of 43 viruses tested in total, were assayed with PTA at pH 6.5, as usually done with crude sap preparations. In 43 of these samples virus particles could readily be detected. Tomato aspermy virus could not be observed at that pH but could at low pH (3.0 to 4.0) as was the case with 6 of 7 isolates (strains) of cucumber mosaic virus and the only strain of alfalfa mosaic virus tested. By accident, tobacco mosaic virus was only tested at low pH but with success. Seventeen isolates were tested with MAT and two of these with MAT only. Results were not better than with PTA pH 6.5 and contrast usually was less. Tomato aspermy virus was detectable with MAT (Fig. 2A) but its effect did not exceed that of PTA at low pH. Five of the 10 isolates that did not contain visible particles when viewed with the electron microscope, did contain virus as revealed in infectivity tests, two did not contain infectivity and three were not tested.

Particles of bean common mosaic virus (Fig. 1C) were easily detected in a newly desiccated bean leaf sample from Morocco, as were particles of cowpea aphid-borne mosaic (Fig. 1B) and of cowpea mosaic virus, yellow strain, (Fig. 2E) in such cowpea leaf samples from Tanzania.

The cowpea mosaic virus from six cowpea samples from Tanzania readily reacted in all cases in agar gel-diffusion tests with antiserum to the yellow strain of the virus up to

Fig. 3. Direct serology in agar gel of cucumber mosaic virus (UK2) from macerated dehydrated leaf material of tobacco $20^1/_2$ years after storage (upper row) and from fresh inoculated cucumber cotyledons (middle row) and systemically infected foliage leaves of cucumber (lower row), both eight days after inoculation. Central wells: antigen, left: undiluted (dry material in buffer 1 to 16 to obtain dry matter content comparable to crude plant sap), right: diluted 1 to 4. Surrounding wells: antiserum, dilutions 1, 2, 4 and 8, respectively.

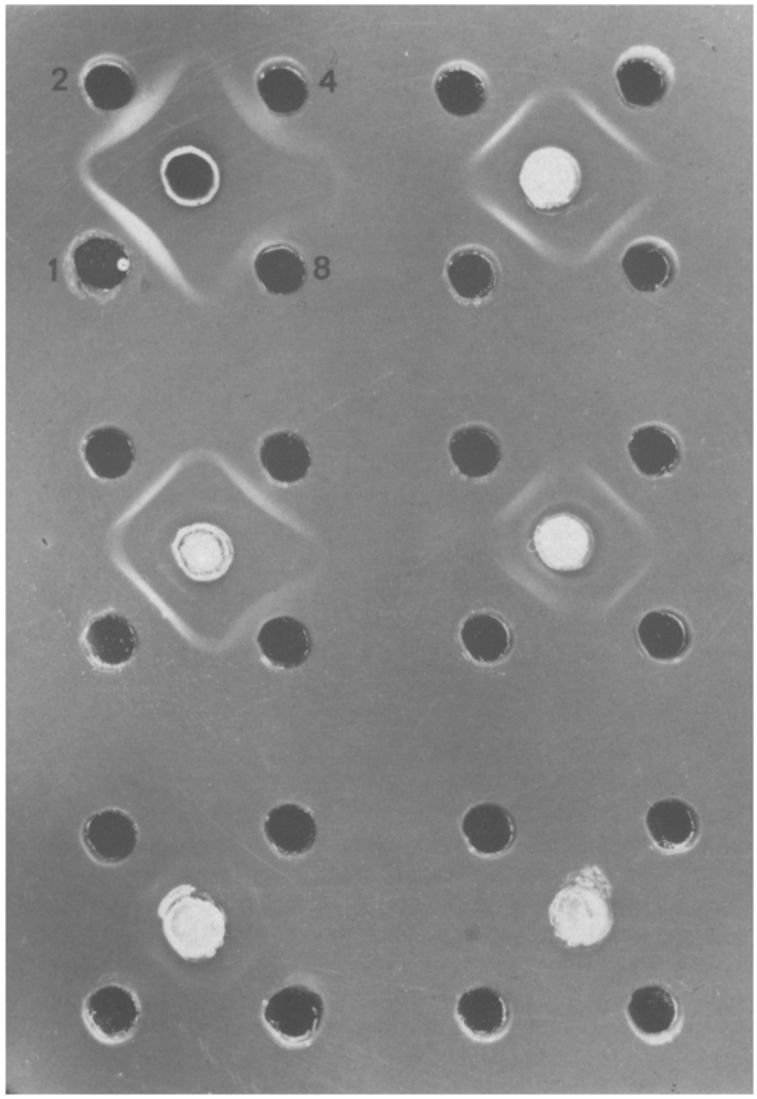

Fig. 3. Directe serologie in agargel met komkommermozaïekvirus (UK2) uit vermalen gedroogd tabaksblad na $20^1/_2$ jaar bewaring (bovenste rij) en uit verse komkommerzaadlobben (middenste rij) en systemisch geïnfecteerde bladeren van komkommer (onderste rij), beide acht dagen na inoculatie. Centrale putjes: antigen; links: onverdund (gedroogd materiaal in buffer 1 op 16 ter verkrijging van droge-stofgehalte vergelijkbaar met ruw plantesap), rechts: verdunning 1 op 4. Omringende putjes: antiserum respectievelijk in verdunningen 1, 2, 4 en 8.

and including antiserum dilution 1024 and with antiserum to the severe strain up to and including antiserum dilution 16.

All serological tests with cucumber mosaic virus isolates B32 (1977-8-16), E31 (1958-9-23), L14 (1959-4-17), UK2 (1959-4-17), vdW (1959-4-17) and ZB2 (1959-4-17) were clearly positive. Most isolates reacted undiluted and diluted 1 to 4 with all four antiserum dilutions (1, 2, 4 and 8). Isolate E31 only did so in undiluted macerate from dry material. The results of comparing macerated dry material with crude fresh virus-containing plant sap and from cucumber are represented in Fig. 3. Sap from inoculated leaves of *C. quinoa* reacted undiluted only.

Broadbean wilt virus (E229, 1973-9-28) was also readily detectable directly serologically in agar 0.85% in 0.05M phosphate citric acid buffer pH 7.0.

Discussion

The results obtained with various viruses (spherical, rod-shaped as well as flexuous) proved that most viruses can be readily detected with the electron microscope in dry leaf material using the same techniques as for fresh leaf samples. Several preparations did contain virus particles in high concentrations. Results with dry samples, even though stored for extended periods of time, usually were not inferior to those with crude fresh leaf preparations.

Application of methylamine tungstate did not yield more reliable results than of PTA pH 6.5. Contrast, however, usually was less. Cowpea mosaic virus, severe strain, was visible with MAT and not with PTA pH 6.5, but this virus usually is easily detectable with the latter stain. Results with single preparations may be erratic. Examination of one, if negative, should be followed by studying a second preparation.

Certain labile viruses, such as alfalfa mosaic virus and cucumber mosaic virus, could more easily or only be detected with PTA at low pH, which is in agreement with results for crude fresh preparations by Milne and Masenga (1978).

Results of direct serological testing of dry material were surprising. Those with cucumber mosaic virus even after $20^1/_2$ years of storage were equal or even better (as with UK2: Fig. 3) than results with fresh material of comparable dilution. They suggest a decrease in concentration of cucumber mosaic virus (UK2) in the following order: (1) dry material of 'Samsun' tobacco $20^1/_2$ years old (Fig. 3, upper row), (2) fresh sap from cucumber cotyledons eight days after inoculation (Fig. 3, middle row), (3) fresh sap from non-inoculated foliage leaves of the same plants (Fig. 3, lower row), and (4) inoculated leaves of *Chenopodium quinoa* six and eight days after inoculation. The impression is that high serological activity not necessarily correlates with high infectivity. This would imply that virus protein may persist in dry material for longer periods or more readily survives desiccation than does intact virus nucleic acid. This is in line with results by Gooding and Tsakiridis (1971) who found that antigenicity of some viruses was preserved longer than infectivity in leaf tissues stored in 1% sodium azide.

Our results with direct electron microscopical assay of dry material stored for varying periods of time up to to $20^1/_2$ years and with a much wider range of viruses extend those of Ragetli et al. (1973) working with freshly desiccated powdered leaf material. The present paper further demonstrates the ease of direct serological assay of desiccated leaf samples for virus infection, even after many years of storage and perhaps even after loss of infectivity.

Both electron microscopy and serology have proved that leaf material with various viruses dried and stored for long periods over $CaCl_2$ may contain viruses in concentrations comparable to fresh material. These observations further stress the value of the preservation technique.

The present results also demonstrate how diagnosis of various sap-transmissible viruses can be performed with plant material, simply dried over $CaCl_2$ in a refrigerator and stored and shipped over $CaCl_2$, at any time and at any place where electron microscopical and serological facilities and expertise are available. Thus biological tests, including those with indicator plants, with attendent risks of virus escape, can be avoided.

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Samenvatting

Rechtstreeks elektronenmicroscopisch en serologisch onderzoek van plantevirussen in boven calciumchloride gedroogd en bewaard bladmateriaal

Zeer uiteenlopende plantevirussen kunnen goed worden bewaard in bladmateriaal van geïnfecteerde planten dat is gedroogd en bewaard boven $CaCl_2$. Bij rechtstreekse elektronenmicroscopische toetsing van 66 monsters met verschillende isolaten van 43 virussen konden in 53 monsters gemakkelijk virusdeeltjes worden waargenomen (Tabel 1 en Fig. 1 en 2). Meestal lukte dit met fosforwolfraamzuur pH 6,5 dat doorgaans voor in ruw plantesap voorkomende virussen wordt gebruikt. Bij luzernemozaïekvirus en komkommermozaïekvirus gelukte dit alleen maar bij lagere pH (3,0 en 4,0). Ook het tomate-aspermievirus was dan veel gemakkelijker aantoombaar. Methylaminewolfraamzuur gaf geen beter resultaat.

Draden van bonerolmozaïekvirus en van 'cowpea aphid-borne mosaic virus' en bolletjes van 'cowpea mosaic virus' waren snel waarneembaar in elektronenmicroscopische preparaten gemaakt van bladmateriaal van recent uit Marokko en Tanzania ontvangen monsters.

'Cowpea mosaic virus', tuinboneverwelkingsvirus en komkommermozaïekvirus konden eveneens gemakkelijk en snel serologisch worden aangetoond in met bufferoplossing vermalen droog blad van respectievelijk 'cowpea', erwt en tabak. Het laatstgenoemde, reeds $20^1/_2$ jaar geleden gedroogde bladmateriaal, bleek in vergelijking met pas geïnoculeerd vers blad van komkommer en *Chenopodium quinoa* zelfs zeer veel serologisch actief virusmateriaal te bevatten (Fig. 3).

De beschreven waarnemingen bevestigen nogmaals de waarde van de toegepaste methode van virusbewaring en tonen aan dat het mogelijk is vele virussen te herkennen in van elders ontvangen gedroogde bladmonsters, zonder het risico te lopen van virusontsnapping, zoals altijd aanwezig bij werk met toetsplanten in de kas.

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