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A virus disease of phaseolus vulgaris L. in the Transvaal

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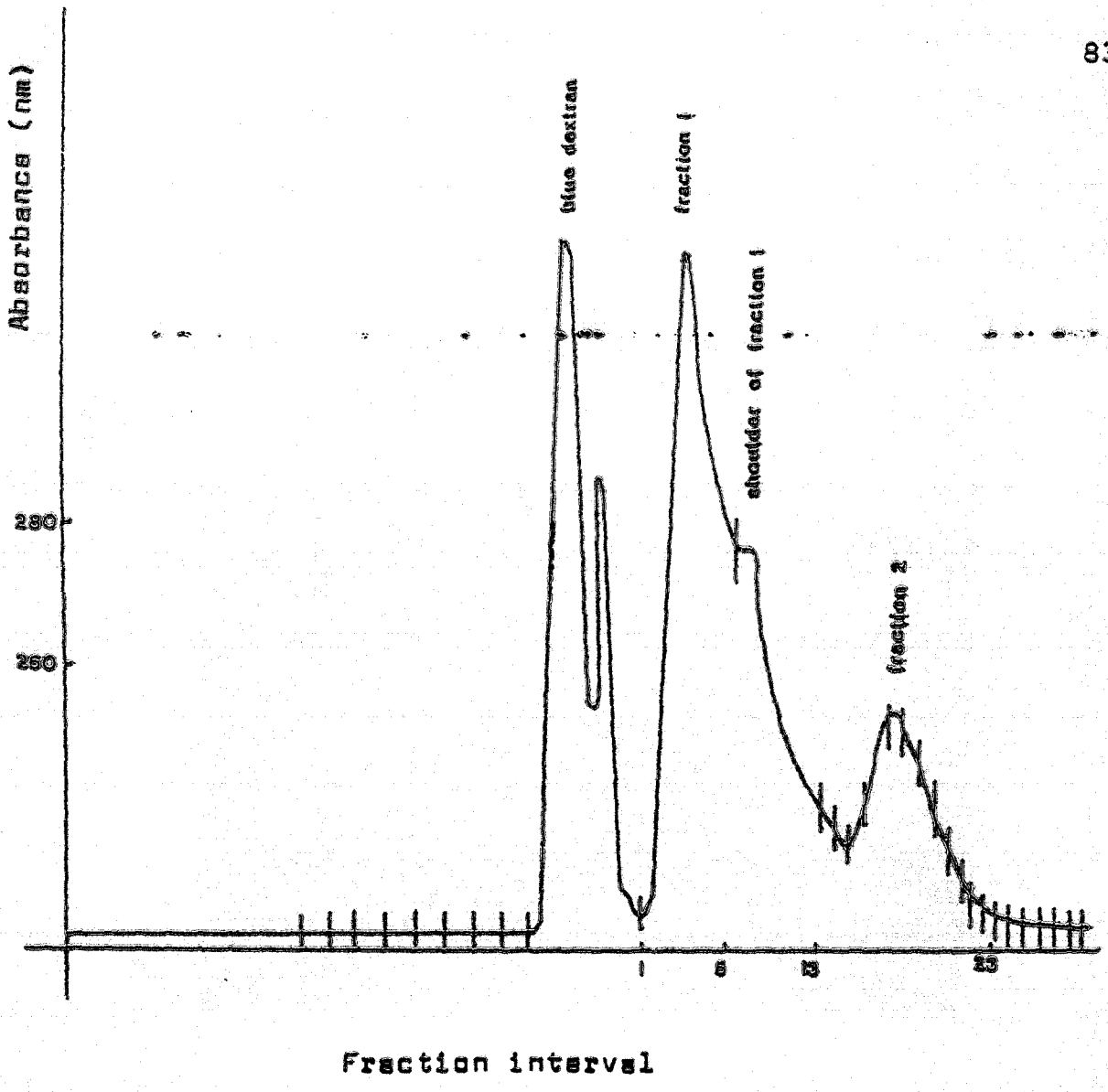


Figure 14: U.V. scan of isolate 247 separated on the glass bead column. Virus could easily be separated from contaminating host components by passing the partially purified sample through the column. High molecular weight host proteins were trapped at the top of the column while low molecular weight components (fraction 1) were eluted before the virus sample (fraction 2 and shoulder of fraction 1). Note that the virus does not elute as a sharp peak but as a curve with a trailing edge, probably caused by fragments of different size eluting sequentially. Electron microscopy indicated the presence of virus particles in the shoulder of fraction 1 and in fraction 2.

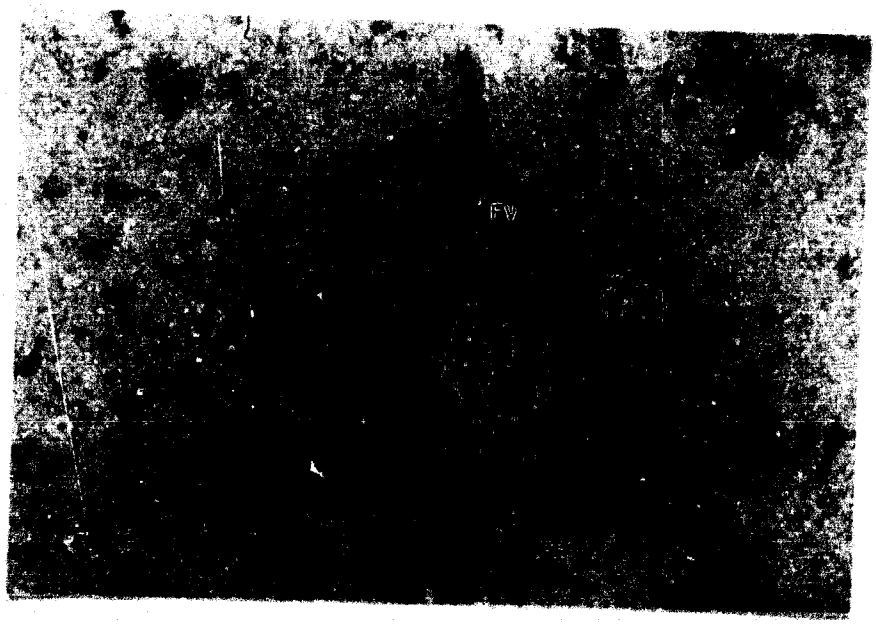


Figure 15: Particles resulting from the separation of isolate 247 by column chromatography. Note the relative absence of both host debris and particle fragmentation (flex virus). The micrograph represents particles found in the shoulder of peak 1 shown in the scan printed in Figure 14.
(x50,000; 2% uranyl acetate stain).

3.6. Serological properties of the presumptive BCMV isolate

The titre of antiserum raised in rabbits against virus purified from sample 247 by procedure 2.3.4. and column chromatography (2.3.5.) was estimated using several serological techniques. The results obtained were compared to ensure an accurate determination of antiserum titre.

All serological tests for plant viruses are a modification of the precipitin test in which antigen combines with its specific antibody to produce an aggregate that scatters light to become visible. To observe precipitation in tubes or on plates requires more material than an indirect measure of combining power such as flocculation of a sensitized particle.

3.6.1. The microprecipitin test

The test was used to follow rising antibody titre in the immunized rabbits. Rabbits were bled between days 21 and 28 of the immunization schedule (2.6.1.) to determine if they had reacted to the virus inoculum. A grid titration, as reported in 2.6.3., was carried out with a dilution series of antigen and antiserum, using very small volumes of both components. Where precipitation occured, it was amorphous and cloud-like, characteristic of rod-shaped viruses (Noordam, 1973). The results of the test obtained after titer stabilization on day 28 are presented in Figure 16. Heavy precipitation was considered 3 plus (+++) and gradations of readings were made to a doubtful precipitate (±).

		antigen (1 mg/ml)									
		1	2	3	4	5	6	7	8	9	10
grid position	1	+++	++	++	++	+	+	+	±	±	-
	2	+++	++	++	++	+	+	+	±	±	-
antibody	3	++	++	++	++	+	+	+	±	±	-
	4	++	++	++	++	+	+	+	±	±	-
	5	+	+	+	+	+	+	+	±	±	-
	6	+	+	+	+	+	+	+	±	±	-
	7	+	+	+	+	+	+	+	±	±	-
	8	±	±	±	±	±	±	±	±	±	-
	9	±	±	±	±	±	±	±	±	±	-
	10	-	-	-	-	-	-	-	-	-	-

Figure 16: Diagramatic representation of the results of the grid titration for the standard microprecipitin test. The titre was 1/256 using this method. No precipitation occured in the control samples, position 10 containing saline in all tests.

KEY:

+++	strong precipitate
++	
+	
±	doubtful precipitate
-	no precipitate

Once the titre was stable and no longer increasing the rabbits were bled by cardiac puncture. Different serological tests were used to determine the final titre of the serum.

		antigen (1 mg/ml)									
		1	2	3	4	5	6	7	8	9	10
grid position	1	+++	++	++	++	+	+	+	±	±	-
	2	+++	++	++	++	+	+	±	±	±	-
antibody	3	++	++	++	++	+	+	+	±	±	-
	4	++	++	++	++	+	+	+	±	±	-
	5	+	+	+	+	+	+	+	±	±	-
	6	+	+	+	+	+	+	+	±	±	-
	7	+	+	+	+	+	+	+	±	±	-
	8	±	±	±	±	±	±	±	±	±	-
	9	±	±	±	±	±	±	±	±	±	-
	10	-	-	-	-	-	-	-	-	-	-

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KEY:

+++ strong precipitate
++
+
± doubtful precipitate
- no precipitate

Once the titre was stable and no longer increasing the rabbits were bled by cardiac puncture. Different serological tests were used to determine the final titre of the serum.

3.6.2. The tube precipitin test

This test was used for the reliable evaluation of the titre of the serum. The range of reactivity of every antigen-antibody system was determined by dilution titration in a 'grid' system. Each dilution of antigen was reacted with each dilution of antiserum. Tubes that presented with heavy precipitation were considered 3 plus (+++) and gradation of readings were made to a doubtful precipitate, that is, a trace reaction ($\pm$). The tube whose contents precipitated most rapidy represented the optimum proportions of antigen and antibody.

Serum titre was determined using the optimum concentration of antigen as a constant and reacting this with various dilutions of antiserum. The antigen dilution chosen for optimum antiserum activity was 1/8. The antibody titre was estimated to be between 1/512 and 1/1024. A slight cross reaction was observed with purified host material at high antibody concentrations (1/8) but not at dilutions of antibody 1/16 to 1/1024. No attempt was made to remove the anithost antibodies from the antiserum even though this is suggested (von Regenmortel, 1966), as only a small quantity of serum was raised which was therefore extremely valuable and as stated, no host reaction was seen when the antibody was diluted.

The results of the tube precipitin test are presented in Figure 17.

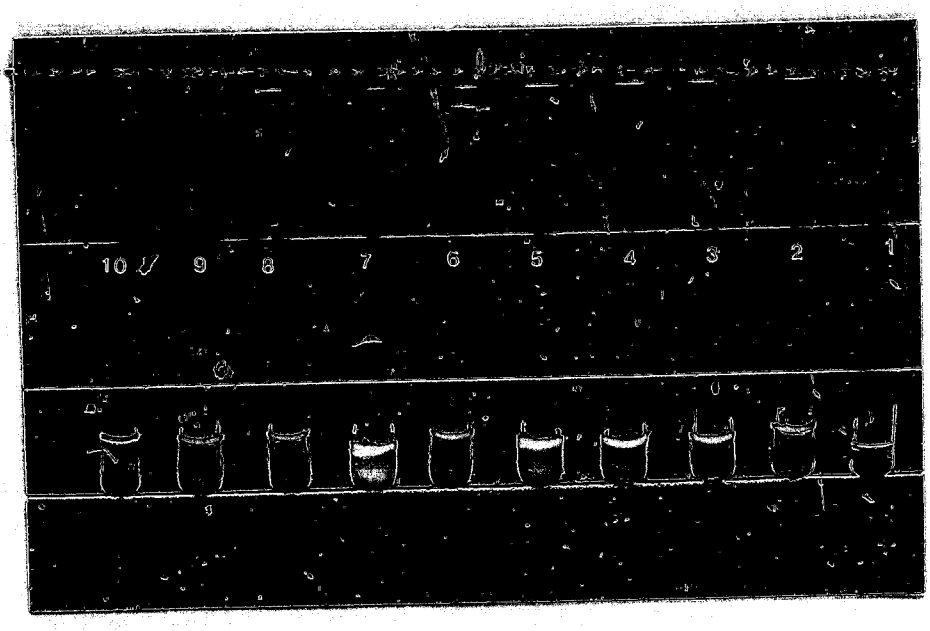


Figure 17: Tube precipitin test using 247 isolate at a constant concentration of 1/8 while varying the raised antiserum concentration from 1/2 to 1/4096. Dilutions of antiserum were made in saline. In the photograph, only those test tubes with a clear precipitation reaction are shown. The dilution factor of each marked tube is presented in the key. No precipitation occured in samples where antiserum was replaced with saline.

KEY:	TUBE	DILUTION
	1	control (saline)
	2	1/2
	3	1/4
	4	1/8
	5	1/16
	6	1/32
	7	1/64
	8	1/128
	9	1/256
	10	1/512

3.6.3. Passive haemagglutination

Titre was low when determined by the two previous methods. This test was selected because of its proposed increased sensitivity in detecting plant viruses and hence its suitability to determine if the titre of the raised antiserum was actually higher than already suggested. With this test, the titre varied between 1/512 and 1/1024 depending on the use of purified or partially purified antigen. The results obtained were therefore similar to other methods despite supposed increased sensitivity.

The results are presented in Figure 18. The test was carried out using varying antigen or antiserum dilutions so that optimal conditions giving the best reaction could be determined.

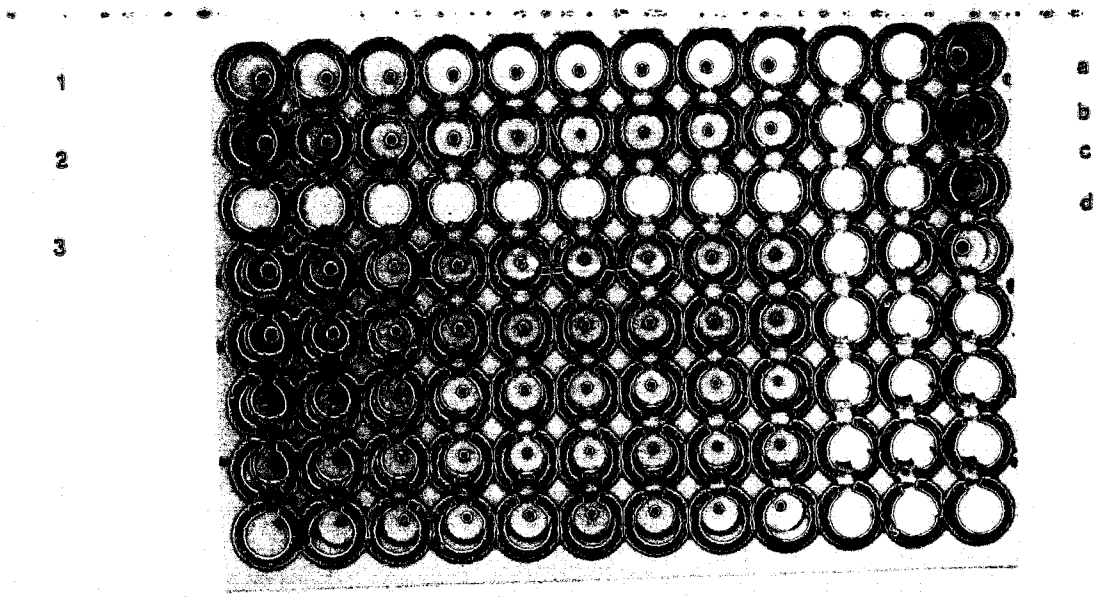


Figure 18: Grid titration of 247 and 259 isolates using passive haemagglutination. Both purified and partially purified samples of virus were used so that the results obtained could be compared. The titre was estimated to vary between 1/512 and 1/1024 depending on the use of purified or partially purified virus. The reaction of healthy sap and sensitized red blood cells (c) however was obscured at lower antibody titres than for previous experiments.

KEY:

Row 1 : 259 purified 1 mg/ml, diluted 1/4 to 1/1024, constant sensitized red blood cells (1/10)

2 : 247 purified 1 mg/ml, diluted 1/4 to 1/1024, constant sensitized red blood cells (1/10)

3 : 247 partially purified, constant concentration (1 mg/ml), sensitized red blood cells diluted 1/4 to 1/1024

Controls a : 0,05 M phosphate buffer, pH 7,2 and sensitized red blood cells

b : 247 purified 1 mg/ml and an equal volume of sensitized red blood cells (1/10)

c : healthy sap and sensitized red blood cells (1/8)

d : healthy sap and unsensitized red blood cells (1/10)

3.6.4. Double diffusion in Ouchterlony plates

Double diffusion techniques can be used to determine the titre of antiserum and relationships of an isolate to other viruses by the use of specific antiserum. Various well patterns are routinely used in these determinations (Hudson and Hay, 1980).
As previously mentioned, gel diffusion can be a problem with flexuous viruses. However, these techniques have been refined by many workers and successful results have been obtained (Gooding and Bing, 1970; Purcifull and Gooding, 1970; Purcifull and Batchelor, 1977; Juretic and Mamula, 1980).

In the current study, the addition of 3% SDS in M/15 phosphate buffered saline, pH 7,2, to the antigen was found to be essential before migration in the gel occured (Figure 19(a)).
Using the different well patterns portrayed in 2.6.6., gel diffusion techniques were satisfactorily employed to determine the titre of the raised antiserum and for the identification of the isolates using supplied BCMV antiserum (obtained from the World Reference Collection, Wageningen, Netherlands).

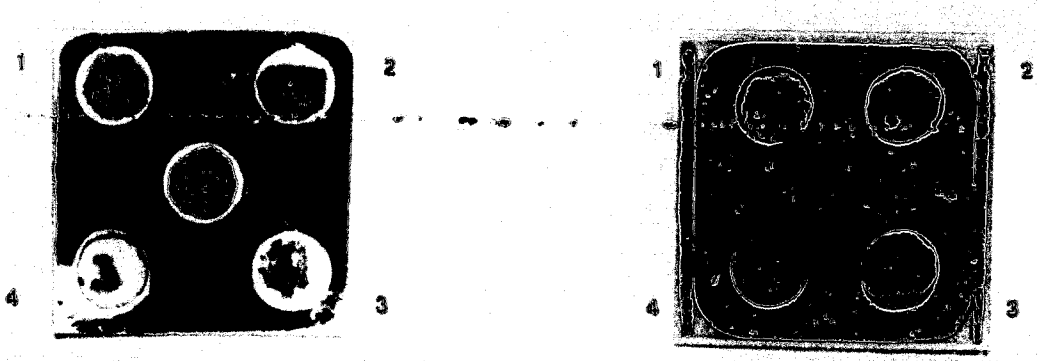
With Ouchterlony plates, the titre of the raised antiserum was found to be much lower (1/32) compared to the other techniques used for titre estimation. The low value may be related to SDS disruption of the virus with the resultant modification of antigenic properties. The reactions of several virus samples to the raised and known BCMV antisera were also investigated. These included known BCMV, PVY and TMV. Both the known BCMV sample and isolate 247 produced a clear line of precipitation when reacted with the supplied BCMV antiserum (from the World Reference Collection, Wageningen, Netherlands). The fact that SDS improved the result is seen in Figure 19(a) and (b). PVY and TMV reacted negatively with supplied BCMV antiserum. The reaction of purified isolate 247 to

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The fact that SDS improved the result is seen in Figure 19(a) and (b). PVY and TMV reacted negatively with supplied BCMV antiserum. The reaction of purified isolate 247 to



a. b.

KEY:

Well	Well
1. healthy purified sap + 3% SDS in M/15 phosphate buffered saline pH 7,2	1. purified 247 (1mg/ml) + 3% SDS in phosphate buffered saline
2. purified 247 (1mg/ml) and the above buffer, no SDS.	2. supplied BCMV antiserum (titre 1/16)
3. Sap 247 and the above buffer, no SDS	3. supplied BCMV antiserum (titre 1/16)
4. purified 247 (1mg/ml) and the above buffer with 3% SDS	4. purified healthy sample + 3% SDS in buffer

Centre: ½ strength raised antiserum

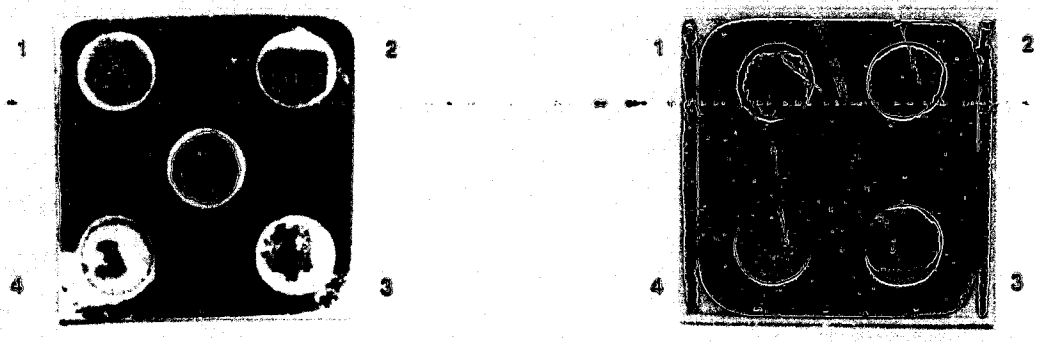
Figure 19: Double diffusion using Ouchterlony plates

(a) Reaction of purified 247 isolate with raised antiserum. Note that the presence of 3% SDS was essential for migration of the antigen to occur so that a line of precipitation was formed.

(b) Reaction of purified 247 isolate with known BCMV antiserum. Note that a line of precipitation occured only with infected material and not with purified healthy material treated in the same way.

Note:

1. The arrangement of wells represented in the figure are only two of the many patterns that were used in these tests.
2. Buffer in all cases is M/15 phosphate buffered saline pH 7,2.



a. b.

KEY:

Well	Well
1. healthy purified sap + 3% SDS in M/15 phosphate buffered saline pH 7,2	1. purified 247 (1mg/ml) + 3% SDS in phosphate buffered saline
2. purified 247 (1mg/ml) and the above buffer, no SDS.	2. supplied BCMV antiserum (titre 1/16)
3. Sap 247 and the above buffer, no SDS	3. supplied BCMV antiserum (titre 1/16)
4. purified 247 (1mg/ml) and the above buffer with 3% SDS	4. purified healthy sample + 3% SDS in buffer

Centre: ½ strength raised antiserum

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Note:

1. The arrangement of wells represented in the figure are only two of the many patterns that were used in these tests.
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to raised antiserum also formed a clear line of precipitation (Figure 19(a)).

Serological investigations using freeze dried sap from survey samples were also performed to determine the identity of the other isolates. Samples 259 and 7 (Potchefstroom) gave positive reactions when sap was reacted with raised and supplied BCMV antiserum. No reactions were observed with the other samples. It is quite possible that low virus concentrations in the initial sap samples used for the tests affected precipitin band formation and hence the negative results cannot be certain.

A very slight host reaction could be observed in gel diffusion studies against raised antiserum (Figure 19(a)), but not against supplied BCMV antiserum (Figure 19(b)).

3.6.5. Immunoelectronmicroscopy

Isolate 247 was further identified as being related to BCMV using immunoelectronmicroscopy with supplied BCMV antiserum (obtained from the World Reference Collection, Wageningen, Netherlands). Three regimes were used to coat grids with virus and antibody (2.6.7.). Regardless of the method used, more particles were seen firmly bound to antibody coated than uncoated grids. The only difference between the methods was seen in the amount of debris that accumulated on the grids. This was reduced by modifying method 1 (2.6.7.) and the introduction of washing stages to remove salts and unbound serum proteins (Figure 20). It is unlikely that debris was accumulated host components as the supplied serum was highly specific for BCMV and unlikely to contain antihost antibodies. Also, control grids using TMV had no particles attached, yet debris was still visible. It is well documented that methodology and charge effects affect the success of immunoelectron-microscopy (Nicolaieff and von Regenmortel, 1980).

To verify the specificity of the reaction, virus at concentrations of 1 mg/ml, 0,5 mg/ml and 0,25 mg/ml were used in a series of tests. By visual observation it was noted that the number of particles on the grids appeared to decrease with low virus concentration.

Serologically specific electronmicroscopy was therefore used as an additional method to identify the 247 isolate as probably being BCMV. The presence of particles on grids is shown in Figure 20.

Virus particles could also be detected in survey samples 259, Witkop, and Potchefstroom 7 using these methods. This provides further evidence that a virus related to isolate 247 was present in these samples. In other survey samples, virus particles could not be detected, even when using method 3 (2.6.7.) which should cause the clumping of virus present in low concentration. Either sufficient virus was not present in these samples or the virus may not have been related to isolate 247.

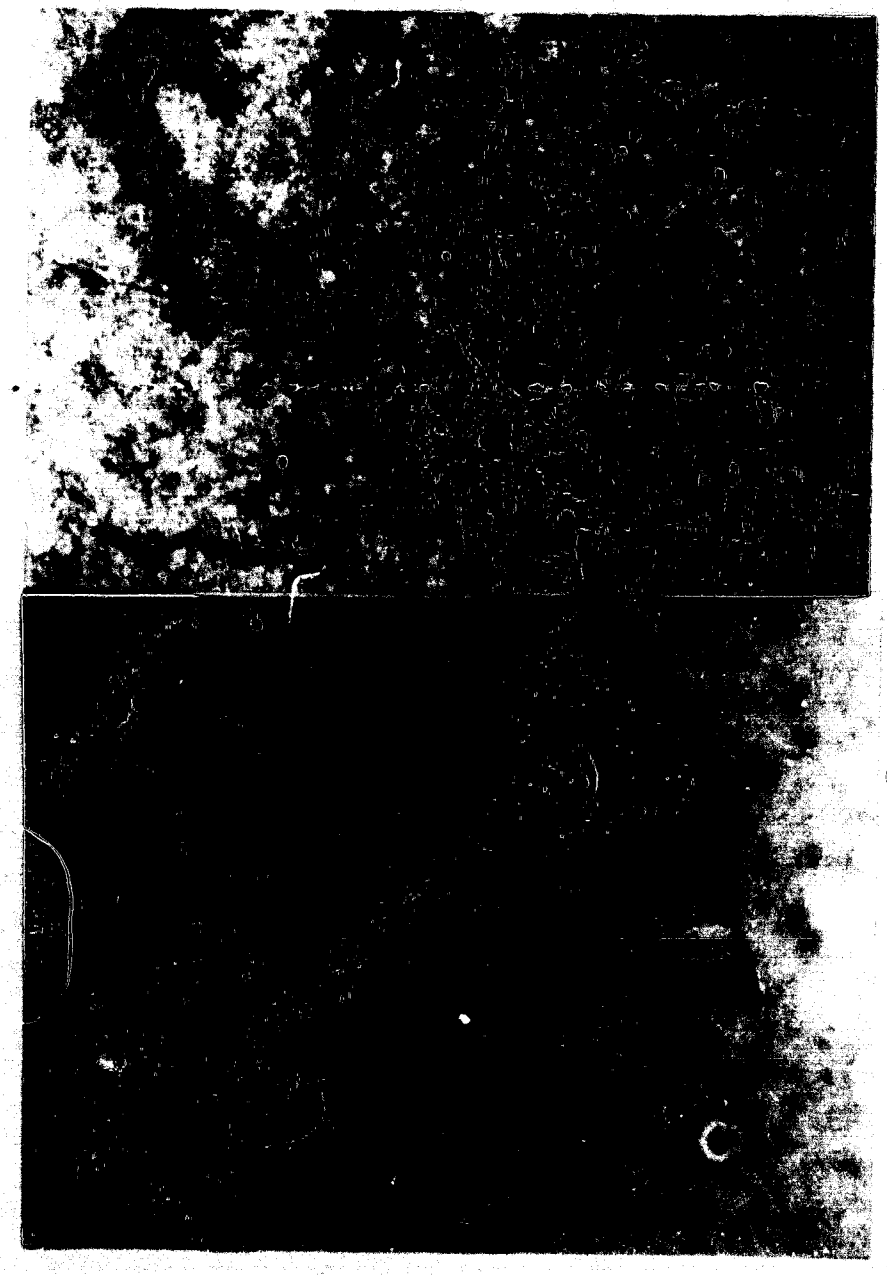


Figure 20: Immunoelectronmicroscopy of 247 isolate.
showing the effectiveness of different methods of serum and virus adsorption

Method 1: grids were floated on a mixture of purified virus (1 mg/ml) and concentrated raised antiserum (1:5½). Virus particles were clearly visible but note the high concentration of debris on the grid. Modifications of this method were made to reduce the amount of debris and to make virus particles more visible.

Method 2: grids were floated on concentrated known BCMV antiserum, then rinsed in PBS, and floated on purified (1mg/ml) virus. This method was used to identify the unknown isolate. Note the increased numbers of particles (flex virus) firmly attached to the grid and the decreased amount of debris.

(x 50,000 for both micrographs; 2% uranyl acetate stain)

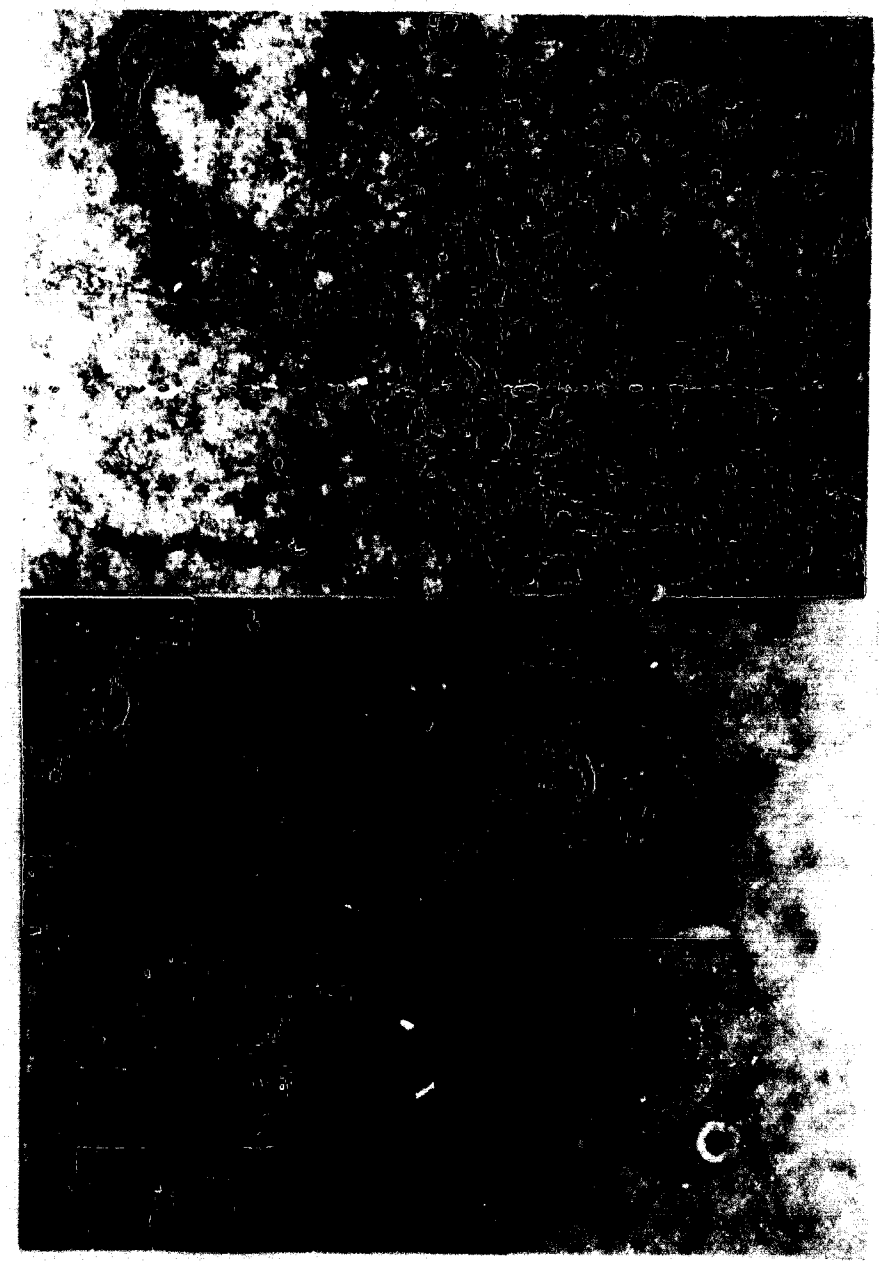


Figure 20: Immunoelectronmicroscopy of 247 isolate.
showing the effectiveness of different methods of serum and virus adsorption

Method 1: grids were floated on a mixture of purified virus (1 mg/ml) and concentrated raised antiserum (1:512). Virus particles were clearly visible but note the high concentration of debris on the grid. Modifications of this method were made to reduce the amount of debris and to make virus particles more visible.

Method 2: grids were floated on concentrated known BCMV antiserum, than rinsed in PBS, and floated on purified (1mg/ml) virus. This method was used to identify the unknown isolate. Note the increased numbers of particles (flex virus) firmly attached to the grid and the decreased amount of debris.

(x 50,000 for both micrographs; 2% uranyl acetate stain)

3.7. Cellular modification of Phaseolus vulgaris var. "Double Dutch Princess" cells infected with the 247 virus isolate.

Infected leaves were removed at weekly intervals after inoculation with virus, and sectioned. During the early stages of infection, modifications in membrane structure frequently occured, whorls of membranes and proliferation of the plasmalemma and cell walls being common (Figure 21(a)). Occasionally, membrane bound crystals could be seen in the cytoplasm of infected cells (Figure 21 (b)). As the infection progressed, groups of "filaments" were seen packed in the cytoplasm of some cells, their dimensions being similar to those of viral fragments (Figure 21(c)). It is possible that they could represent viral aggegates as it is documented that potyvirus particles often occur in parallel arrays, scattered throughout the cytoplasm of infected cells (Hollings and Brunt, 1981).

In the advanced stages of infection, modifications in chloroplast structure occured. Starch grains accumulated and granal and stromal contents appeared to disintegrate (Figure 21(d)). Pinwheel inclusions, a diagnostic feature of members of the PVY group, were not seen in infected cells. Cylindrical cytoplasmic inclusions (thought to represent pin wheels in longitudinal section (Christie and Edwardson, 1977)) were seen (Figure 21(a)). No alterations in cellular structure or cytoplasmic content were seen in healthy leaves sectioned in the same way.

3.8. Molecular properties of the 247 isolate

3.8.1. Estimation of the molecular weight of viral protein

Purified virus consistently produced two bands on acrylamide gels, irrespective of acrylamide concentration or virus purification procedure used. These bands were

Figure 21: Cellular modifications in Phaseolus vulgaris var. "Double Dutch Princess" infected with the 247 isolate

a) Cells showing cylindrical cytoplasmic inclusions (CCI) and wall proliferations (WP). (x 20,000; 2% uranyl acetate + lead citrate)

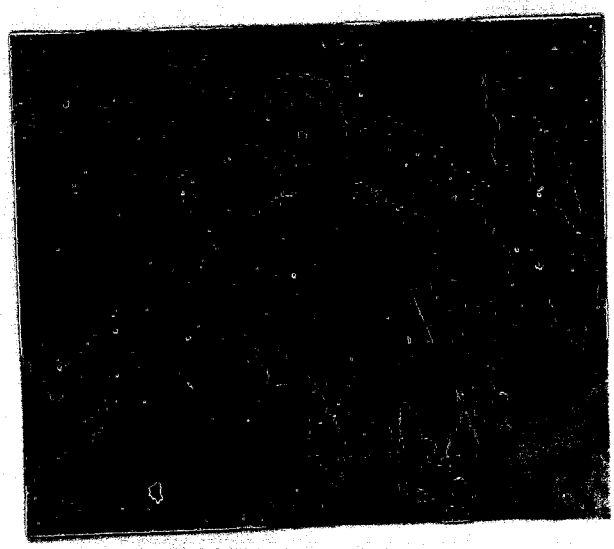
b) membrane bound crystal (c) seen in the cytoplasm of an infected cell. (x 50,000; (2% uranyl acetate + lead citrate)

c) groups of "filaments" (f) that could represent viral aggregate in infected cell cytoplasm (x 15,000; 2% uranyl acetate + lead citrate)

d) modifications in chloroplast structure during the advanced stages of infection. Note accumulated starch grains (s.g.) and disintegrated stromal and granal contents. (x 10,000 and 8,000; 2% uranyl acetate + lead citrate).



a.



b.



c.



a.

not seen when purified material from healthy samples was electrophoresed in the same way.

The glass bead column method (2.3.5(b)) resulted in virus of highest purity and when such samples were electrophoresed host components producing high molecular weight bands on gels, were eliminated. To determine the molecular weights of the virus specific bands, samples were electrophoresed against markers of known molecular weight (Pharmacia, Uppsala, Sweden).
A separation of protein components can be seen in Figure 22. The high and low molecular weight components (38,000 and 29,000 daltons respectively) can be distinguished from the remaining bands. The molecular weights were determined graphically after comparison with standards of known molecular weight (Figure 23).

3.8.2. Estimation of the molecular weight of viral RNA

Of the different methods used to extract viral RNA, the phenol-chloroform method, proposed by Perry et al. (1972), was the most successful. A virus concentration of 1 mg/ml yielded 29,26 ug of RNA and this was divided into samples before electrophoresis. The separation of RNA species is shown in the U.V. scan from a gel stained with ethidium bromide (Figure 24). The molecular weight of the viral RNA band was determined by comparison with standards of knwon molecular weight. A graph of the results obtained is presented in Figure 25. Viral (247) RNA was found to have a molecular weight of $3,5 \times 10^6$ daltons.
An attempt was made to visualize the RNA preparation to determine its structure, this method being successfully used in animal virology (Coetzee and Pretorius, 1979). Referring to Figure 26, RNA can be seen to be a single strand with occasional convoluted regions. No studies have been made on RNA of bean viruses but it is likely that BCMV and related viruses have single stranded RNA as is found for other potyvirus group members (Hollings and Brunt, 1981).

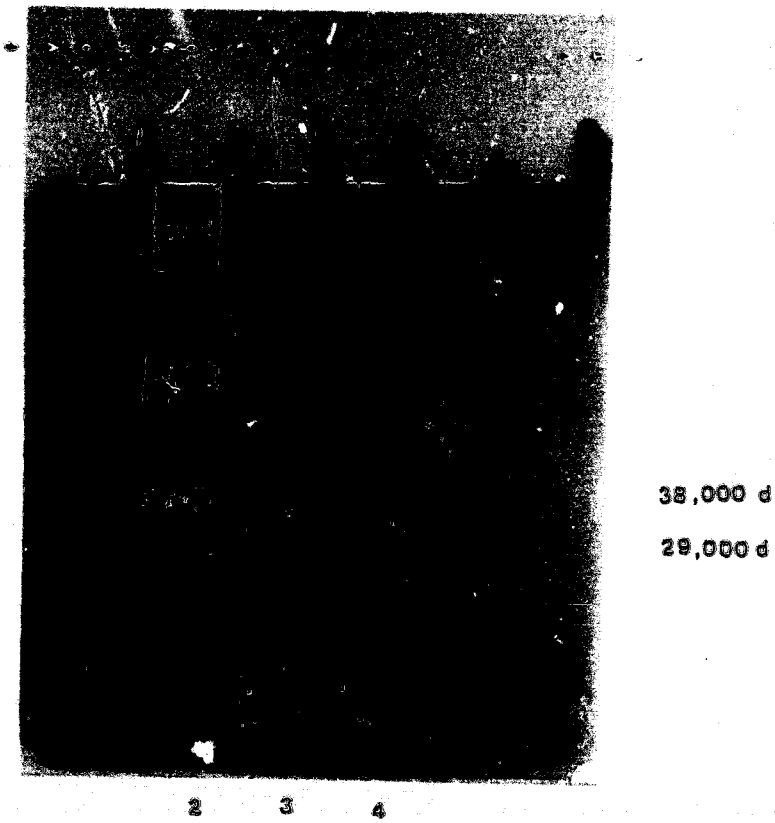


Figure 22: 7% acrylamide gel after electrophresis of isolate 247 coat protein. Virus was purified by column chromatography and concentrated to 1 mg/ml by ultracentrifugation. Virus was then treated with splitting solution and electrophoresed with standard marker proteins. Infected samples consistently showed bands of 38,000 and 29,000 daltons.

KEY:

Well 2 marker protein
3 247 sample
4 247 sample

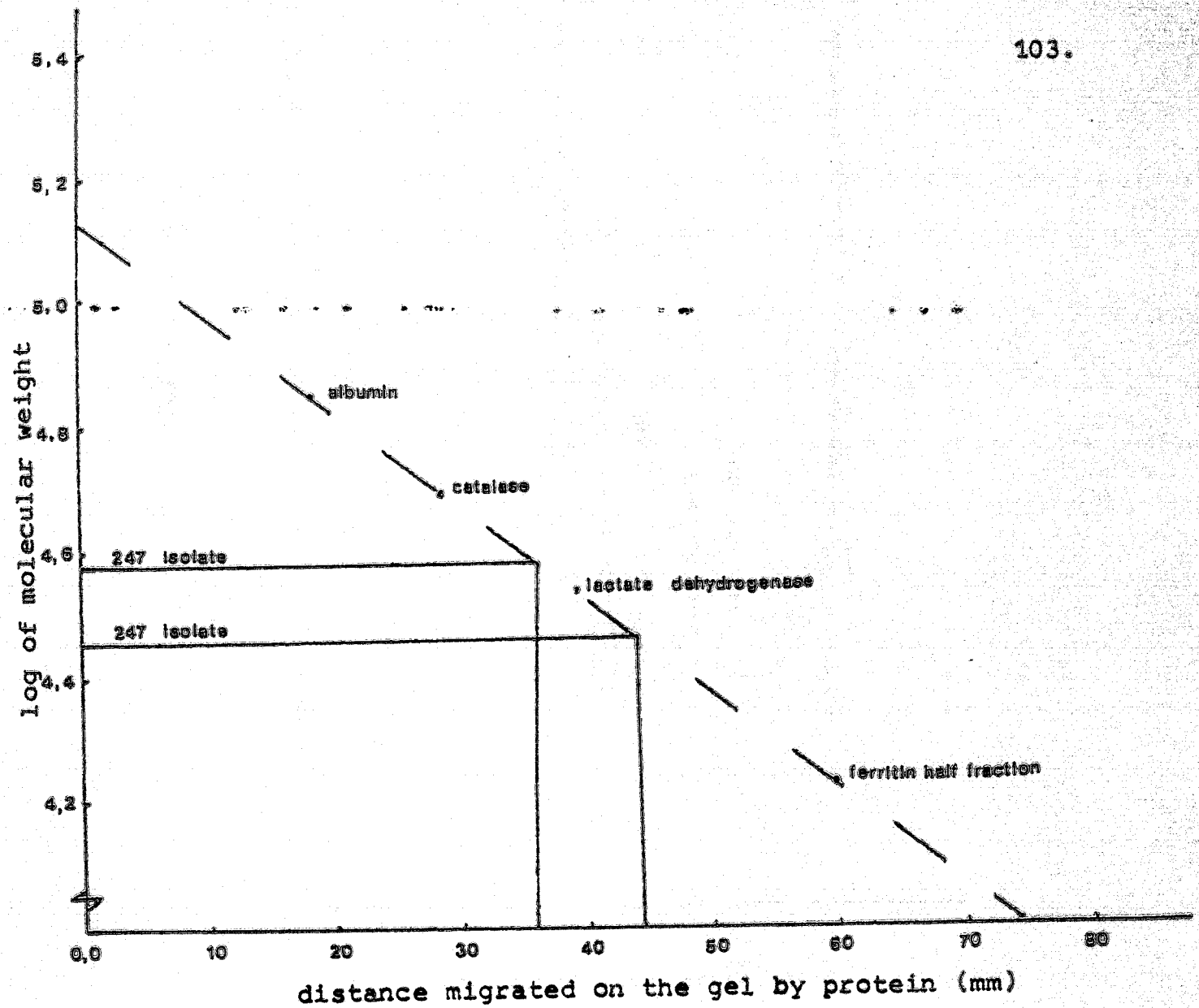
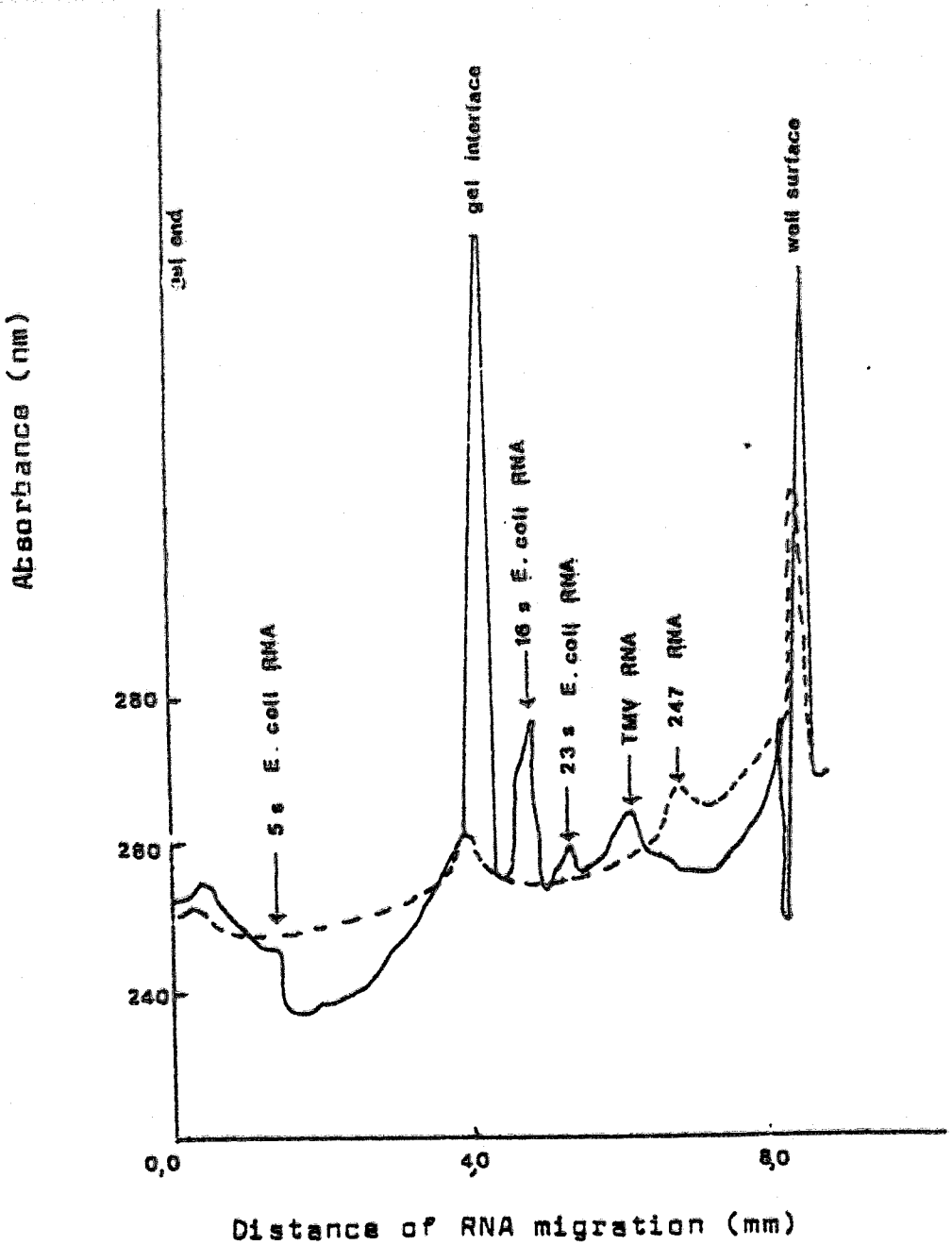


Figure 23: Estimation of the molecular weight of the protein component of isolate 247

The $\log_{10}$ of markers of known molecular weight was plotted against distance migrated by the proteins (in mm). In this way a standard curve was constructed from which the unknown molecular weights of the viral protein bands could be determined.



Molecular weights: TMV $2,0 \times 10^6$ d

E. coli 23s $1,1 \times 10^6$ d

16s $5,6 \times 10^5$ d

247 $3,5 \times 10^6$ d

Figure 24: U.V. scan of separated RNA species after electrophoresis on RNA acrylamide gels

RNA was extracted by the phenol-chloroform method of Perry et al. (1972). RNA bands were scanned at 254 nm after staining with ethidium bromide.

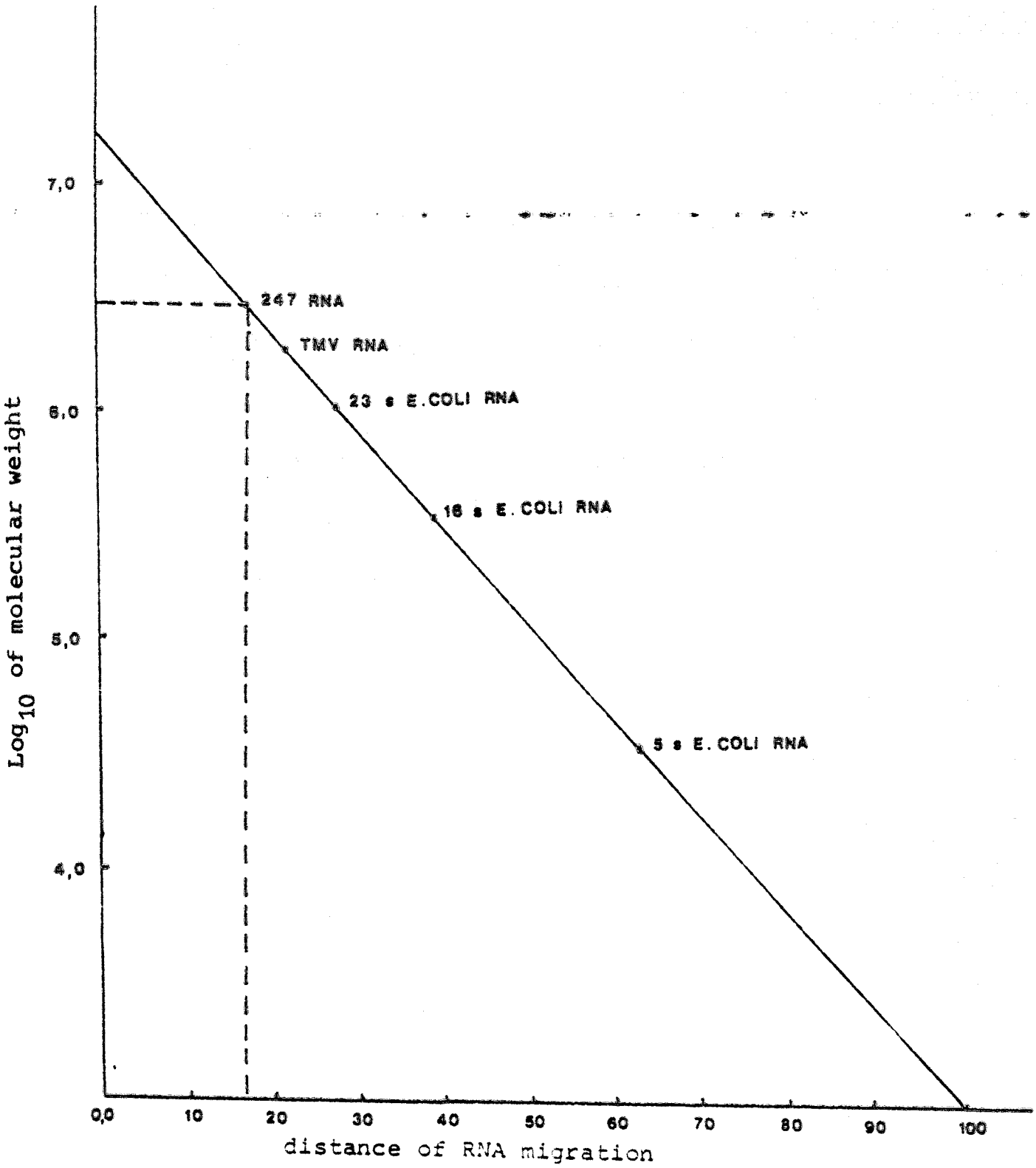


Figure 25: Estimation of the molecular weight of 247 RNA
A standard curve was constructed by plotting the $\log_{10}$ of known molecular weights against distance migrated on the gel (in mm). From the standard curve, the molecular weight of 247 RNA was estimated to be $3,5 \times 10^6$ daltons.

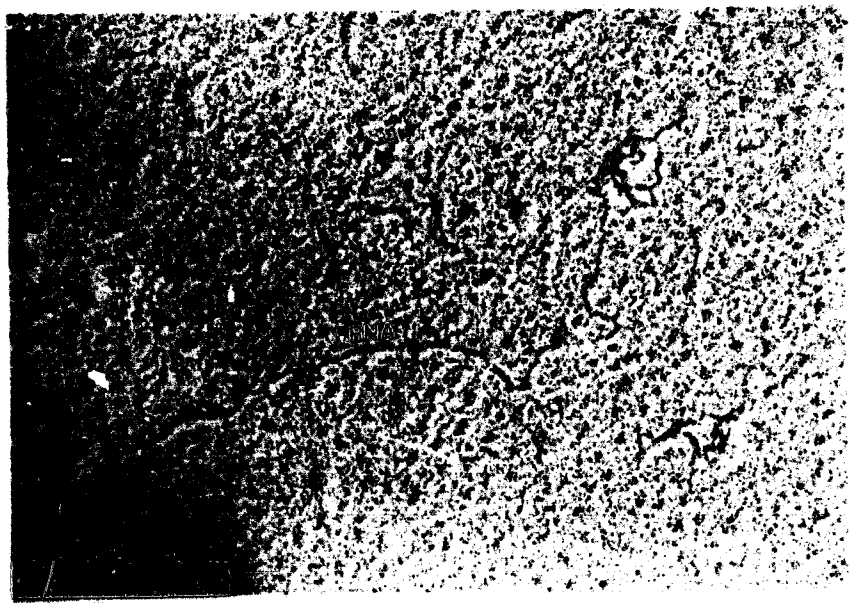


Figure 26: Electron micrograph of purified 247 RNA

Visualization of RNA was carried out to determine strandedness (x 50,000; platinum shadowing).

3.9. Strain determination

From the indicator species, gel diffusion and immunoelectron-microscopy studies, it appeared as if isolate 247 was likely to be BCMV. It therefore seemed important to ascertain which strain of virus the isolate closely resembled. Although the most reliable method of strain determination is by serological reactions with different strain specific antisera, this could not be performed in the current study as sera were unavailable worldwide. For this reason, the well documented method of Drijfhout (1978) was followed to determine the strain of BCMV in the study. The work done by Drijfhout (1978) using differential hosts for the determination of BCMV strains solved the confusion at the time and until strain specific sera become available, will remain the most appropriate method.

The results of inoculations on a differential host range of *Phaseolus vulgaris* varieties suggest that the strain of virus may be related to NL5 (Table 12). The range of symptoms produced were similar to those documented for NL5 (Drijfhout, 1978), although certain differences were apparent. Cultivars of Groups 3 and 4 were considered tolerant because the differential hosts showed no symptoms, whereas mosaic has previously been recorded for NL5. Group 6 also reacted differently, the isolate causing local lesions on *Phaseolus vulgaris* var. "Monroe", which does not occur with strain NL5. Reactions in all other groups (Figure 27) however correlated well with those documented for NL5.



Figure 27: Symptoms on Phaseolus vulgaris varieties after infection with 247 isolate.

a) Group 1: Phaseolus vulgaris var. "Comptesse de Chambord" showing local lesions (LL)

b) Group 3(a): Phaseolus vulgaris var. "Wintergreen" showing extensive systemic black vein necrosis (BVN)

Table 12: Reaction of cultivars of *Phaseolus vulgaris* to isolate 247

Host genotype group	*Phaseolus vulgaris* varieties	Host reaction	Symptoms produced 2 weeks post-inoculation
1	Diacol calima	+ S	yellow systemic mosaic
	Canellini	+ S	leaf curl
	Comptesse de Chambord	+	chlorotic local lesions
2	Bataaf	+ S	leaf curl, mosaic
	Redlands Greenleaf C	+ S	slight leaf distortion
3	Redlands Greenleaf B	- T	no reaction
	Great Northern 1140	- T	no reaction
	Colombia Pinto	- T	slight yellowing
4	Sanilac	- T	no reaction
	Pinto UI 111	+ T	slight leaf curl
5	Pinto 114	+ S	leaf curl, yellowing
6	Monroe	+ H	large brown lesions
8	Cornell 49 - 242	$^{+}$n H	black veinal necrosis
	Nep II	$^{+}$n H	yellowing, vein necrosis
	Seafarer	$^{+}$n H	plant death
9 (a)	Jubila	$^{+}$n H	plant death
	Wintergreen	$^{+}$n H	black vein necrosis
9 (b)	Topcrop	$^{+}$n H	black vein necrosis
10	Amanda	$^{+}$n H	yellowing, death

KEY: + susceptible, systemic mosaic
$^{+}$n susceptible, systemic necrosis
- no systemic symptoms
T tolerant cultivars
S sensitive cultivars
H hypersensitive cultivars

3.10. Seed screening programme

To ascertain if the raised antiserum could be used to detect the presence of virus in seed, the direct ELISA technique was used. Specific IgG was purified from the raised antiserum and its identity proven using immuno-electrophoresis (against anti-IgG) and polyacrylamide gel electrophoresis. When electrophoresed on 7% protein gels, split IgG was found to separate into a high molecular weight band of 50,000 daltons and a low molecular weight band of 25,000 daltons. When electrophoresed without splitting solution, IgG formed a single high molecular weight band of 150,000 daltons. Immunoelectrophoresis of concentrated IgG against anti-rabbit IgG resulted in a distinct line of precipitation (Figure 28). Purified IgG was used to coat microtitre plates for the ELISA. In addition, IgG was conjugated with alkaline phosphatase enzyme required for the direct ELISA to be performed.

Initially a grid titration was carried out (Figure 29) from which it was obvious that wells treated with healthy sap reacted similarly to the other controls of PBS-Tween while rows A and B treated with infected material gave a stronger reaction. As limited amounts of raised antiserum were available and as the healthy sap did not react in the test to any greater extent than buffer alone, the serum was not cross-adsorbed before use.

Considering the concentration of purified IgG used for coating the wells, it was obvious that 10 µg/ml gave the strongest reaction and the greatest difference in intensity between infected material and the controls.

As the ELISA test had to be visually assessed, it was important to have strong colour development. Although a 1/800 dilution of conjugated IgG gave a specific reaction with the infected sap, a 1/200 dilution gave a deeper



Figure 28: Immunoelectrophoresis of purified IgG on 2% agarose gels in barbitone buffer, pH 8,2. IgG was purified using the standard rivanol/ saturated ammonium sulphate procedure. A distinct line of precipitation could be seen after staining gels in 0,2% Coomassie blue in methanol and water.

KEY: Wells : specific IgG
Troughs: anti-rabbit IgG

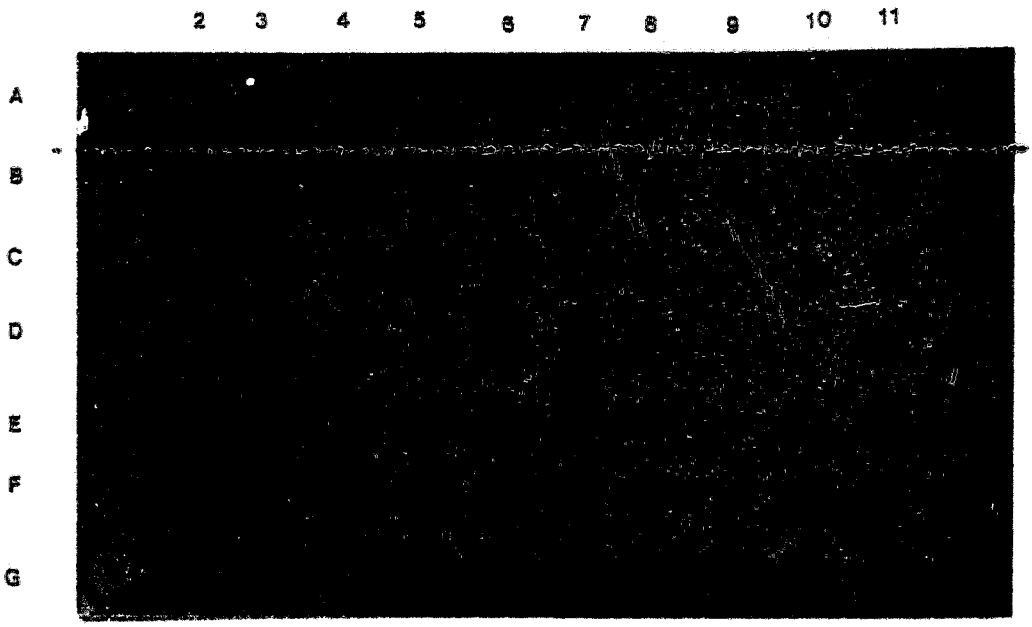
and more readily observable reaction. Hence for further studies plates were coated with IgG at 10 µg/ml and a 1/200 dilution of conjugate was used.
The weak reactions seen in rows E and F may have been due to inefficient washing of the plates between reaction stages. This could obviously be improved by washing the microtitre plates more than 3 times between steps.

In a second experiment, the specificity of the antiserum was tested by comparing the reactions of sap from plants infected with isolate 247 with that of TMV infected plants and purified TMV. No difference in the intensity of the reaction occured when TMV, sap from TMV infected bean plants or PBS-Tween alone was reacted with coated wells. A distinct reaction only occured when isolate 247 was used as the antigen source.

The ELISA technique was then extended and adapted to test for virus in bean seed lots, some of which were suspected of harbouring BCMV. The seeds were various cultivars of Phaseolus vulgaris but known healthy seed was not available for each cultivar tested. The controls used were sugar bean seed, virus free, (supplied by Professor B. von Wechmar) and Phaseolus vulgaris var. "Double Dutch Princess" supplied by Plant and Seed Control. The seed was allowed to germinate for 48 hours prior to use in the ELISA test, and where appropriate dissected into its component parts. Twenty germinating seeds of each lot were used in each test. An intense colour reaction was recorded as presumptive evidence of virus and given a 3 plus (+++) rating. The different regions of the seed reacted differently (Table 13). Although a weak reaction was obtained with control seed, it was similar in intensity to that where PBS-Tween alone was used in the test and this was recorded as a negative reaction (-).

A complete summary of all reactions of the seed lots tested is presented in Table 13. An extremely good relationship was observed between colour intensity (presumptive evidence of virus presence) and biological assay of the seed lots, performed by inoculating each seed lot onto Phaseolus vulgaris "Bataaf", a variety that reacts systemically to BCMV infection.

The results of this preliminary study showed that the raised antiserum was capable of detecting virus in seed and that the embryo from germinating seed may be the more reliable component to check for the presence of virus in any BCMV seed screening programme.



KEY:

A concentrated infected sap
B 1/10 infected sap diluted with PBS-Tween
C 1/100 infected sap diluted with PBS-Tween
D 1/1000 infected sap diluted with PBS-Tween
E, F healthy sap, control
G PBS-Tween 20 only

2,3,4 : 10 µg/ml coating
5,6,7 : 1 µg/ml
8,9,10: 0,1 µg/ml
2,5,8 : 1/200 dilution of conjugate in PBS-Tween
3,6,9 : 1/800 dilution of conjugate in PBS-Tween
4,7,11: 1/3200 dilution of conjugate in PBS-Tween

Figure 29: ELISA grid titration: purified IgG, conjugate and sap at different dilutions.

Table 13: Comparison of seed assay results using the ELISA technique and inoculation of indicator plants

Seed Lot	Elisa test of seed components				Indicator inoculation
	whole seed	coat	embryo	cotyledon	
APC 98E31 P1	+++	++	++	+	+
Various lines E	-	-	-	-	-
PC 976 9EA	±	±	±	-	-
Yellow haricot	-	-	-	-	-
DP 3	-	-	-	-	-
Pinto U1 114A	-	-	-	-	-
Bonus Col BCMV	±	-	+	-	+
DP 8 & &	±	±	+	-	+
P 10 D	-	-	-	-	-
PC 98261 78/79	-	-	-	-	-
A PC 97E25 P1	-	-	-	-	-
A PC 84C7	-	-	-	-	-
B PC 9734 E1	-	-	-	-	-
PC 84 C8	-	-	-	-	-
A PC 9427 P2	-	-	-	-	-
PC 97-21-E1	-	-	-	-	-
PC 84C1 A	++	++	++	++	+
A PC 9417 HZ	+	+	++	+	+
A PRETO 145	+	+	++	+	+
SUGAR BEAN	-	-	-	-	-

Numbers of replicates: duplicate

Number of seeds used : 50 seeds per sample were soaked - 20 germinating seeds were selected for the test and were ground in PBS-Tween buffer before use in the ELISA

($\frac{20}{50}$ = 40% germination)

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DP 3	-	-	-	-	-
Pinto U1 114A	-	-	-	-	-
Bonus Col BCMV	±	-	+	-	+
DP 8 & &	±	±	+	-	+
P 10 D	-	-	-	-	-
PC 98261 78/79	-	-	-	-	-
A PC 97E25 P1	-	-	-	-	-
A PC 84C7	-	-	-	-	-
B PC 9734 E1	-	-	-	-	-
PC 84 C8	-	-	-	-	-
A PC 9427 P2	-	-	-	-	-
PC 97-21-E1	-	-	-	-	-
PC 84C1 A	++	++	++	++	+
A PC 9417 HZ	+	+	++	+	+
A PRETO 145	+	+	++	+	+
SUGAR BEAN	-	-	-	-	-

Numbers of replicates: duplicate

Number of seeds used : 50 seeds per sample were soaked - 20 germinating seeds were selected for the test and were ground in PBS-Tween buffer before use in the ELISA

($\frac{20}{50}$ = 40% germination)

CHAPTER 4

Discussion and Conclusion

Samples collected were subjected to several tests, that is, inoculation on a host range, purification and electron microscopy. Of all the samples collected from the farms only those obtained from Witkop, Potchefstroom and Ermelo produced symptoms on the indicator plants (Table 8). Indeed, these samples showed viral symptoms on the leaves when the material was originally collected from the farms. The plants obtained from Witkop were severely stunted and yellowed, while other plants showed milder symptoms confined to the leaves and were primarily a form of leaf yellowing or mosaic.

Leaf material of sample 9 from Potchefstroom was the only sample showing unusual symptoms when collected. The symptoms were typical of those traditionally caused by mycoplasmas, such as stunting, proliferation of branches and small leaves. Symptoms however were produced on indicator plants implying the presence of a pathogen that could be sap transmissible, such as a virus rather than an MLO. No particles however could be detected in leaf dips or after purification of the leaf material.

Leaf dips using sap from infected leaves failed to show the presence of viral particles for most of the samples, possibly due to low virus concentrations. Virus particles were only detected in expressed sap of 4 samples (Table 10). Sample 7 from Potchefstroom was severely infected and flexuous rods were seen under the electron microscope (Figure 3). The particles measured 750 nm in length and 15 nm in width. These values closely approximate those obtained by Bos (1971) for BCMV. An identical particle, in terms of morphology and dimensions was isolated from infected leaves of samples 247 and 259 grown from seed as

well as from leaves of the propagation host (Figure 9). A spherical particle was seen in expressed sap from the Pretoria sample (Figure 2). The diameter was approximately 26 nm.

When leaf samples were purified however, 4 additional samples showed the presence of viral particles (Table 10), supporting the idea that the concentration of virus in the infected material was not sufficient to enable particles to be detected in the expressed sap. A total of 8 possible virus isolates were therefore obtained, of which 7 were flexuous forms and 1, a small spherical particle. It was extremely difficult to measure the additional 4 isolates as particles became severely fragmented in their isolation (Figures 3, 4 and 5). Width measurements however, were consistently in the range of 13 to 15 nm and the particles tended to show a flexuous morphology. No attempt was made to characterize each isolate as the original symptoms, indicator studies and electron microscopy suggested that the isolates, especially of Group II (Table 9), were similar, or possibly strains of the same virus. Hence detailed studies were performed on one isolate only (247), large quantities of seed infected with this isolate being available. As one of the aims of the current study was to determine if the ELISA technique could be used to detect virus in bean seed, this isolate appeared to be the most appropriate for further purification and antiserum production.

The spherical particle was tentatively identified as TNV on the basis of host range inoculations, morphology and preliminary investigations of biological properties (Table 8). The virus is extremely well characterized (Kassanis, 1970) and is also not an example of a seed borne pathogen in beans. For these reasons, the properties of the virus were not further investigated.

After inoculation of 247 onto a range of specific host plants (Table 11), results indicated that the symptoms produced were typical of the potyvirus BCMV (Table 14). In general, potyviruses have restricted host ranges and induce mosaic or mottle symptoms in leaves (Hollings and Brunt, 1981).

The properties of the purified virus from this sample supported the tentative identification of a potyvirus. A range of purification procedures was used including general methods (2 and 3 (2.3.)) and procedures optimized for BCMV purification (4 (2.3.)), the latter being the most successful method for the isolation of virus, not only from sample 247, but also from other samples, again suggesting that the flexuous isolates may be of a similar nature.

On the basis of these preliminary investigations, therefore, isolates 247, 259 and Potchefstroom 7 were tentatively identified as BCMV and this was later confirmed using serological techniques (3.6.5.; Figures 19 and 20). Further studies on the 247 isolate were performed in an attempt to characterize it and develop methodology for detecting its presence in seed.

The best propagating host was found to be Phaseolus vulgaris var. "Double Dutch Princess" (Figures 6 and 7) in which the isolate produced mosaic and attained a high enough concentration for purification. By comparison to other systemic viruses however, the yield of virus per 100 grams of infected leaves was low, therefore several hundred grams of infected material was essential before purification could be carried out. Leaves were collected 13 to 15 days after inoculation and the virus purified immediate

When purifying elongated viruses there are two main difficulties to overcome: aggregation and fragmentation (Huttinga, 1973). Of all the purification procedures used, procedure 4 (2.3.4.) promoted by Jafarpour et al., (1979) gave the highest yield of virus and the best purity.

Several methods, such as that proposed by Morales (1979) for BCMV were totally unsuccessful, the use of high concentration (0,5M) potassium phosphate buffer in the initial purification step resulting in the precipitation of phosphate and virus at 4°C, the temperature at which all procedures are carried out. It was then impossible to resuspend the samples. Potassium phosphate buffers form the basis of most purification procedures for BCMV but the molarity should not exceed 0,05 M when working at 4°C.

Sap extracts were best clarified with an equal volume of cold chloroform which removed most plant components and pigments. After centrifugation, virus was precipitated from clarified extracts using PEG (MWT 6,000) and sodium chloride, 7% (w/v) PEG being used instead of the recommended 8% (Jafarpour et al., 1979), so that the pellets could be more readily resuspended.

Indeed, Huttinga (1973) has suggested that concentrations of as little as 2 to 4% PEG may induce severe aggregation, making resuspension of virus impossible in small amounts of buffer. However the use of PEG circumvents the problem of virus concentration by several cycles of ultracentrifugation which causes breakage of flexuous rods, although as stated, resuspension after precipitation may be incomplete (Hamilton et al., 1981).

Purification buffer and pH may also affect solubility (Huttinga, 1973), although in the current study, a moderate pH buffer system was used (2.3.4).

Several problems were encountered when purifying the 247 isolate. Like most elongated viruses, it had a tendency

to aggregate side by side or end to end, and resuspension fragmented the particles (Figures 9, 11 and 13), making measurement difficult. Yield of virus was reduced, probably because of adsorption to cell debris, the sedimenting of BCMV with chloroplasts being well documented (Bos, 1971).

Purification procedures for potyviruses are increasing, but the general conclusion from most reports seems to be that even with complicated procedures, only small amounts of virus can be otained and that methods for purifying one virus cannot generally be applied to other members of the same group. The previous lack of information relating to chemical properties of potyviruses may be attributed to difficulties in purification. Until an ideal means of obtaining a pure sample can be found, research in this field is restricted. Also, owing to the brief longevity in vitro (3.4.), isolate 247 could not be stored for long periods as it disintegrated into components. When virus was required for any experimental procedure therefore, it had to be rapidly purified and used immediately.

For the determination of physiochemical properties and the production of antiserum, highly purified preparations were required and additional steps in purification had to be used to eliminate contaminating host components.

Differential centrifugation produced virus preparations of sufficient purity for use in electron microscopy, gel diffusion and other serological procedures. Purity was increased with the use of gradients and column fractionation. Sucrose gradients were found to be more convenient than caesium chloride, and obviously less expensive with much shorter centrifugation times. There were however several problems. The osmolarity of the sucrose solutions, even when prepared in resuspension buffer (Appendix I) damaged intact virus particles. It was

essential to dialyse or centrifuge out the excess sucrose from pure preparations or virus tended to dissociate which made its recovery impossible.

Caesium chloride gradients provided extremely pure virus samples (Figures 12 and 13) as opposed to sucrose gradients (Figures 10 and 11). In caesium gradients host components were found to separate as a band of higher density above the opalescent virus band therefore contamination was reduced. The buoyant density value of virus in caesium chloride was 1,27 g/cm^3 which was slightly lower than the average value for members of the potyvirus group (1,31 g/cm^3), suggested by Hollings and Brunt (1981).

The use of both types of gradients was not without difficulty. Yield losses occured with fractionation of the virus band and as only small amounts of virus could be separated on these gradients, the method was inefficient.

Purification problems seemed to be overcome with the use of the controlled pore glass bead column. Partially purified virus could easily be separated from host components after passing through the column. High molecular weight host proteins were trapped at the top of the column while low molecular weight components were eluted before the virus sample (Figure 14). BCMV did not elute as a sharp peak, characteristic of isometric and other small viruses, but as a curve with trailing edges. The curve may be related to virus structure as the elongated particles invariably become fragmented during the purification procedures.

Other advantages of column fractionation were that:

a) larger volumes (1,0 - 1,5 mls) of partially purified virus could be applied, as opposed to 0,5 mls for gradients, and the concentration of debris in the

sample had little effect on virus purity and resolution of bands. Virus was extremely well separated as seen in the U.V. absorption profile (Figure 14) and from electron microscopy on pelleted fractions (Figure 15).

b) Chromatography using CPG totally avoids damage by gravitational fields and osmotic shock, yielding a product suitable for electron microscopy or other tests without the need to remove gradient material (Barton, 1977). Concentration by ultracentrifugation yielded a pure virus product.

c) Several separations could be done on one column, a brief time interval separating each one, without the fear of contamination. The separation profiles of healthy samples were compared with those obtained from virus infected material. The peak known to be that of viral particles was not present in healthy scans.

Pure virus required in the chemical characterization tests was therefore prepared using the glass bead column as it was found to be the most efficient method of purification.

Biological properties of isolated virus were also used as a guide in identification. Estimations of thermal inactivation point(T.I.P), dilution end point (D.E.P.) and longevity *in vitro* (L.I.V.) of the isolate were first made using *Phaseolus vulgaris* var. "Beka". The T.I.P. was between 55°C and 60°C, the D.E.P. between 10^{-3} and 10^{-4} and the L.I.V. was one day. Symptoms on inoculated leaves appeared as necrotic local lesions that rapidly coalesced. Ideally, host plants selected for biological assays should show local lesions that are clear enough to be counted. The symptoms expressed on *Phaseolus vulgaris* var. "Beka" were not totally satisfactory so the test was repeated using detached leaves of a highly susceptible local lesion host, *Chenopodium quinoa* (Noordam, 1973). The 247 isolate produced diffuse chlorotic local lesions,

about 2 mm in diameter on inoculated leaves, but at high virus concentration only. Leaves started to yellow and die before the test could be completed. It was impossible to obtain any conclusive results and here it is clear that intact plants seemed essential for such assays.

The test was repeated using Phaseolus vulgaris var. "Monroe" as several studies observed consistent production of local lesions on primary leaves following mechanical inoculation with BCMV (Saettler and Trujillo, 1972; Trujillo and Saettler, 1972; Meiners et al., 1978). As stated previously, preliminary investigations suggested that the 247 isolate was BCMV.
Necrotic local lesions appeared on primary leaves about one week after inoculation (Figure 8(a)). These gradually enlarged, developing into characteristic ring-spot type lesions, accompanied by limited vascular necrosis (Figures 8(b) and (c)). Lesion number appeared to be directly proportional to dilution factor of inoculum therefore results were based on visual observations and no quantitative data on lesion number was recorded. Results obtaind were also similar to those values for the isolate assayed on Phaseolus vulgaris var. "Beka". The T.I.P. was between 50° and 60°C, the D.E.P. ranged between 10^{-3} and 10^{-4} and the L.I.V. was one day. In all experiments, control plants showed no symptoms.

In general, the results for the biological properties correspond to those documented for BCMV by Bos (1971). The T.I.P. is usually about 60°C but this value can vary depending on virus source, strain and environmental conditions. The D.E.P. is between 10^{-3} and 10^{-4} and sap retains infectivity at room temperature for 1 to 4 days.

Immunoelectronmicroscopy and double diffusion techniques in agar were used to confirm the identity of the 247

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Immunoelectronmicroscopy and double diffusion techniques in agar were used to confirm the identity of the 247

isolate known BCMV antiserum (prepared against BCMV and supplied from the World Reference Collection, Wageningen, Netherlands) was used in all experiments. The antiserum had a titre of 1/16.

Using Ouchterlony plates, a distinct line of precipitation occured when purified virus (1 mg/ml of the 247 isolate) was reacted against supplied BCMV antiserum (1/16) (Figure 19(b)). Freeze dried samples of the remaining isolates were also tested against supplied BCMV antiserum. When reacted, only samples 259 and 7 (Potchefstroom) showed a line of precipitation similar to that obtained with 247 indicating that the viruses present were probably also BCMV. All other samples failed to show a precipitation reaction.

Double diffusion tests were at first confined to isometric viruses that diffuse easily through agar. Rods and filaments greater than 600 nm in size always aggregated and were only able to diffuse once treated with SDS so that chemical dissociation of the virus into components occurs. In spite of a great deal of controversy, several workers have shown that SDS-treated flexuous virus may be detected with double diffusion tests (Gooding and Bing, 1970; Purcifull and Gooding, 1970; Uyemoto et al., 1972 and Purcifull and Batchelor, 1977). It should be pointed out that only in cases where a great deal of disparity exists between whole virus and its SDS-denatured coat protein need an antiserum to the latter be made to achieve immunodiffusion results (Purcifull and Batchelor, 1977). Indeed the new Western blotting procedure routinely used in many laboratories relies on recognition by antibody of antigens separated in an SDS-gel system.

For immunoelectronmicroscopy, formvar grids were always used despite reports by Derrick and Brlansky (1976) that serum proteins do not stick. The supplied BCMV antiserum

was used for coating grids and virus particles from the 247 sample were seen firmly attached, methodology affecting only the concentration of background debris (Figure 20). The rinsing of grids between reaction stages (2.6.7.) provided a clearer background and particles were more easily visible (Figure 20).

Freeze dried samples from the survey were tested using the most complex IEM method (2.6.7(3)) and supplied BCMV antiserum. Particles were detected on grids only in samples 259, 7 (Potchefstroom) and 1 (Witkop). Results again indicate that these samples contained a similar virus to that of the 247 isolate. Other samples may have contained different BCMV strains or even different viruses and therefore gave no reactions with Ouchterlony plates or IEM. The specificity of the trapping reaction would in all probability have been due to BCMV antibodies as normal serum coated grids had no virus particles attached. In addition, the number of attached particles appeared to increase with increased virus concentration.

The use of concentrated antigen preparations and PBS washing steps gave the best results and method 3 (2.6.7.) increased clarity as particles became surrounded by a halo of antibody, forming a more visible complex.

It should be noted that only one set of experimental conditions was tested. Variations in reaction times, dilutions of serum and temperatures for incubating samples are factors that could influence the numbers of particles attached to grids (Derrick, 1973).

To summarize, it was found that:

1. washing of the grids, as described in 2.6.7. (2) and (3) removed excess salts, non specific proteins, cell debris and impurities but did not disturb the virus/ antibody complex.
2. failure to wash grids resulted in a granular background and crystaline deposits
3. all methods gave good resolution, more virus always being detected than in conventional leaf dips
4. the incubation times and dilutions chosen were arbitary and manipulation of these conditions may possibly have given better results
5. the inclusion of the "decoration step" (2.6.7.(3)) increased the width of particles making them easier to see.

IEM was used to confirm the identity of the 247 isolate. This technique is extremely sensitive, quick and reliable for the identification of unknown viruses. It has been well documented by Thomas (1980) that IEM can be used in place of other serological methods, such as the ELISA, for virus detection and identification, IEM being up to three times more sensitive. IEM was selected to identify the isolate for practical reasons, such as limited serum availability. It confirmed the tentative results from the Ouchterlony plates, indicator and biological property studies already discussed. Hence it appears that the 247 isolate is BCMV and that samples 259, 7 (Potchefstroom) and 1 (Witkop) may also be BCMV.

The viral nature of a pathogen is not completely established unless it has been physicchemically characterized. Emphasis therefore falls on molecular structure, especially of the coat protein and nucleic acid (Bos, 1978).

In the chemical characterization, isolated virus protein and nucleic acid components were subjected to polyacrylamide gel electrophoresis to determine their molecular weights.

In the protein determinations, the "short treatments" of sap samples described by Paul (1974) were unsuccessful. The presence of virus bands could only be detected after careful and thorough purification of virus from host components. Infected material consistently possessed two protein bands, a low molecular weight component of 29,000 daltons and a high molecular weight component of 38,000 daltons (Figures 22 and 23). These bands were not found in healthy samples treated in exactly the same way. The bands appeared irrespective of the purification procedure followed, or the concentration of acrylamide used in the gel. Results obtained show some correlation with previous reports on the protein components of BCMV (Moghal and Franki, 1976) and other potyviruses (Hattinga and Mosch, 1974). Most workers have recorded a heavy and a light component, varying in concentration depending on the virus. Morales (1979) however suggests the presence of three protein subunits of 29,000, 32,000 and 34,000 daltons respectively, while Uyemoto et al.(1972) detected a single band of 35,000 daltons. Generally, values reported for BCMV are much higher than for other potyvirus members but there is little agreement about the number of protein components present (Moghal and Franki, 1976), or their origin.

Gough and Shukla (1981) have recently commented on the apparent heterogeneity of potyvirus coat proteins. The high molecular weight component is now generally accepted to be that of capsid protein while several explanations have been proposed about the origin of the other bands. Hollings and Brunt (1981) have suggested that proteolytic enzyme degradation of protein may account for the low molecular weight components as these increase in concentration with storage of virus. Hill and Benner (1980) proposed,

on the basis of amino acid analysis, that viruses like BCMV dissociate into stacking rings of different sizes. They suggest that these components of different sizes may account for the electrophoretic patterns obtained.

The high molecular weight value obtained for this isolate could also reflect a strain difference. No comparisons on the molecular weights of the protein from different BCMV strains have been made.

Peptide maps and amino acid analysis may give a better indication of coat protein properties as considerable variations obtained with SDS-PAGE have led to some confusion about potyvirus molecular weights. It was hoped that better results for protein gels could be obtained by increasing the sensitivity of the staining technique for protein. The photographic staining method of Merril et al. (1981) however proved unsuccessful, as gels were probably beyond the specified thickness required for chemical penentration.

Even less information is known about the genome properties of potyviruses, especially BCMV. Of the few viruses studied that are representative of the group, the nucleic acid comprises 5 to 6% of the total particle, and is generally a single stranded RNA molecule, having a molecular weight in the region of $3,0 \times 10^6$ daltons (Leakle and Pares, 1977). In the current study, the molecular weight of isolated RNA was estimated at $3,5 \times 10^6$ daltons. No comparison can be made with the literature as BCMV RNA has not been investigated. The RNA from a structurally similar particle, TEV, has a molecular weight of $3,2 \times 10^6$ daltons (Hari, 1981) which is not dissimilar to the value obtained experimentally for 247 RNA in the current study (Figures 24 and 25).

The phenol:chloroform procedure (Perry et al., 1972) gave the greatest yield and purity of RNA in spite of reports that viral RNA can be sensitive to phenol (Bruering, 1972). Discontinuous gel systems (Petri, 1972) gave the best separation of RNA species. The best stain was ethidium bromide, as RNA bands were more clearly visible than when using methylene blue, although gels had to be scanned at U.V. wavelengths (254 nm) which may not always be as sensitive as visible wavelengths (Aron, 1980). Nevertheless the RNA bands were always sufficiently clear and easily detected when gels were scanned at U.V. wavelengths.

Electron microscopy was used to visualize the structure of RNA (Figure 26). Such techniques are successfully used to study nucleic acid structures of animal and human viruses (Coetzee and Pretorius, 1979). RNA strands appeared as collapsed single stranded threads yet the use of formamide helped retain some secondary structure. The aim was simply to show the single stranded nature of the isolated RNA. This could also have been done by electrophoresis of RNA on a series of gels of different acrylamide concentrations. Such methods however require considerable amounts of purified RNA and hence were not practical in the current study where large amounts of plant material would have been required.

Another property associated with potyvirus infections is the formation of distinct membranous and other inclusions in the infected cells. BCMV is known to form distinct cytological entities that can be used for the rapid identification of infection (Edwardson, 1974). Cylindrical inclusions were seen in the cytoplasm of infected cells (Figure 21(a)) but characteristic "pinwheel" inclusions were not seen. It is possible that the cylindrical structures represented transitional forms such as laminated plates. The cytoplasm showed other changes however,

characteristic of a potyvirus infection, such as wall proliferations and the presence of possible viral particles (Figures 21(a), (c) and (d).

Crystal inclusions were present in the cytoplasm (Figure 21(b)). These are thought to be induced as a consequence of altered cell metabolism with infection and are usually non viral structures. Changes in metabolism account directly for the alterations in cytoplasmic content. Organelles become modified, chloroplasts for example becoming amorphous masses due to the degeneration of grana. Structural modifications of cell walls and plasmalemma occured frequently. Membranes proliferated and combined often with vesicles of wall material to form wall proliferations or paramural bodies.

In general these are the changes that were seen in BCMV infected cells.

The literature suggests that there are several <u>strains</u> of BCMV and there has always been a great deal of confusion and misunderstanding related to their identification. As there are no basic differences in the common mosaic symptoms expressed by the strains of BCMV, a strain may be characterized by two main methods:

1. using serological tests such as the ELISA, with known antisera to the different BCMV strains. This is obviously the most sensitive and the most precise way of strain determination, yet this method cannot always be used. The availability of the antisera is the determining factor.
2. By the reaction of the isolate on several <u>Phaseolus vulgaris</u> cultivars, that is, determination of its pathogenicity spectrum. This was the method selected to determine the strain of BCMV due to the lack of strain specific antisera being available.

Initially test hosts used in each country differed, so interpretations were questionable. Recently however, an internationally acceptable and standard range of differential cultivars for the identification of BCMV strains has been developed (Drijfhout, 1978). Strains have now been differentiated and classified on the basis of reactions on these differential hosts.

From the host reactions documented in Table 12 and Figure 27, the strain was assigned to Group VI of the scheme described by Drijfhout (1978), (Table 15). The host reactions of cultivars in Group 8 are most important in this respect as only Group VI strains cause a systemic reaction in all plants at the temperature range (22°C to 30°C) used. The host reactions of cultivars in Group 10 distinguish clearly between the two strain representatives of Group VI. Phaseolus vulgaris var. "Amanda" only reacts to strain NL5 and not to NL3. The two strains can only be distinguished on this basis as reactions are identical for all other groups.

Results of the current study indicate that the 247 isolate caused host reactions most similar to those symptoms induced by strain NL5. However there are some differences when results obtained from this isolate are compared with the literature. Host Groups 3 and 4 were considered tolerant because differentials showed no real symptoms, whereas Drijfhout (1978) recorded a systemic mosaic for NL5 with these host groups, and hence he considered these cultivars to be susceptible. The results obtained for host Group 6 also differed; the reactions on "Monroe" were those of local lesions followed by a limited degree of necrosis around the lesions, therefore the cultivar was considered susceptible. Drijfhout had not recorded systemic symptoms and considered the cultivar resistant. Results however correlated directly with those he obtained for NL5 inoculated onto cultivars of Groups 8 to 10. No other virus strain recorded causes systemic necrosis in every

member of these groups except NL5. The unknown isolate could also be distinguished from strain NL7 on these host reactions. Hence it appears that the isolate may be a new strain or it may be possible that two strains were present in the infected seed causing the host reactions recorded and not one single strain at all. The strains previously thought to occur in South Africa are NL7 and NL8 (Vermeulen, pers. comm.).

The results of the current study suggest that NL5 or a totally new strain of BCMV may recently have been introduced and that this new strain was responsible for the symptoms produced in sample 247 in 1978.

One major problem encountered when using the strain system proposed by Drijfhout (1978) is that it does not allow for the detection of mixed infections. A more sensitive technique such as the use of serological reactions could possibly alleviate this problem and it remains possible that sample 247 was infected with more than one strain.

The main features of the isolate studied in detail in the current research are presented in Table 14. A comparison is made between the properties of the isolate and features characteristic of the potyvirus group and the BCMV type strain.

Seed transmission is now known to be an important dispersal mechanism for several viruses (Shepherd, 1972). Reddick and Stewart (1919(a) and (b)) were the first to show that BCMV was seed borne in different varieties of Phaseolus vulgaris. As much as 50% of the progeny may be diseased depending on the time of infection and environmental conditions. The success of seed transmission is now recognised to be an indirect cause of BCMV epidemics. Randomly distributed infected seed are sources of disease when plants emerge from the soil and the timing is right for virus spread. Such seed borne sources of virus are extremely important in the case of BCMV, epidemics frequently resulting as the established virus is efficiently spread

Table 14: The main features of isolate 247 as determined in the present study. The main features of the Potyvirus group and BCMV Type strain are provided for comparison

FEATURES	ISOLATE 247	POTYVIRUS GROUP	BCMV TYPE STRAIN
Characteristics	flexuous particles, 750 nm x 13 nm; buovant density in CsCl of 1,27 g/cm^3, 2 molecular weight protein bands of 29,000 daltons and 38,000 daltons, single stranded RNA genome of molecular weight 3,5 to 10^6 daltons, T.I.P. 50°C to 60°C, L.I.V. 1 day, D.E.P. 10^{-3} to 10^{-4}, induce mosaic and mottle symptoms, pod and seed malformations, 247 has a host range restricted primarily to Leguminosae members, transmitted mechanically in sap and in seed	flexuous, filamentous particles, 720-900 nm x 11 nm, buoyant density in CsCl of 1,31 g/cm^3, ± 2 protein subunits, molecular weight range 32 to 34 x 10^3 daltons, enclosing single stranded RNA genome of molecular weight 3,0 to 3,5 x 10^6 daltons. T.I.P. 50°C to 75°C. L.I.V. 1 to 50 days, D.E.P. 10^{-1} to 10^{-6}, induce mottle and mosaic symptoms, restricted host ranges, transmitted mechanically in sap and by aphids, some in seed	flexuous, filamentous particle, 750 nm long x 15 nm wide, no data recorded for properties of the particles or for particle composition. T.I.P. 60°C, variable with virus source and the environmental conditions, L.I.V. 1 to 4 days at 20°C, D.E.P. 10^{-3} to 10^{-4}, virus causes common mosaic with leaf malformations and rolling, and black root necrosis type symptoms in certain cultivars of Phaseolus vulgaris. Severity of symptoms varies with the environmental conditions, cultivars and time of infection. Readily transmissible to many members of the family Leguminosae and Chenopodiaceae. The virus is also transmitted by seed, pollen, aphids and sap
Geographical distribution	Transvaal, Lowveld and Highveld areas have recorded infections, Cape Province	occur world wide but are more prevalent in tropical and sub-tropical countries	worldwide
Cellular relations	crystalline inclusions, modified mitochondria, vesicles, membrane proliferation, transitional laminated plates and possible viral particles seen in infected cell cytoplasm	Induce the formation of conical or cylindrical inclusions in the cytoplasm. Some members induce crystalline and nuclear inclusions	cytoplasmic inclusions occur in epidermal strips especially in chlorotic areas of infected bean leaves

FEATURES	ISOLATE 247	POTYVIRUS GROUP	BCMV TYPE STRAIN
Serological relationships	not determined	complex; most are related to one or many members of the Potyvirus group Serological comparisons have been proposed as a criterion for the separation of this large group into subgroups	serologically related to several members of the Potato virus Y group
Ecology	not determined	a successful group of pathogens, flourishing in a wide group of crops under diverse environmental conditions. Different factors affect their rate of spread and their severity in crops	in nature, this pathogen is restricted mainly to Phaseolus species and is probably common wherever these crops are grown

Table 15:

Resistance spectra (rows) of the host differentials and pathogenicity spectra (columns) of the strains compared to 247 in this study

Resistance group of the host	Differential cultivar	Pathogenicity group and virus strain										
		I	II	III	IV		V		VI		VII	
					a	b	a	b	a	b		
		NL 1	NL 7	NL 8	NL 5	NL 6	NL 2	NL 2	NL 3	NL 5	NL 4	
Cultivars with recessive alleles (I^+I^+) of the necrosis gene												
1	Dubbele Witte	+	+	+	+	+	+	+	+	+	+	
2	Imuna	-	+	-	+	+	+	+	+	+	+	
3	Redlands GrB	-	-	-	+	+	-	-	+	+	+	
4	Michelite	-	-	+	-	-	+	+	+	+	-	
5	Pinto 114	-!	-	-	-	-	+	+	+	+	-	
6	Gr North J1	-	-	-	-	-	-	-	-	-	+	
Cultivars with dominant alleles (II) of the necrosis gene												
8	Widusa	-	-	+n	-	+n	-	-	+n	+n	-	
9a	Jubila	-	-	-	-	+n	-	+n	+n	+n	-	
9b	Topcrop	-	-	-	-	+n	-	+n	+n	+n	-	
10	Amanda	-	-	-	-	-	-	-	-	+n	-	

(Drifthout, 1978)

KEY:

\+ susceptible, systemic mosaic

+n susceptible, systemic necrosis

\- no systemic symptoms

by insect vectors (Jafarpour et al., 1979).

BCMV therefore has the potential for rapid spread once established and as the primary source of infection is through seed, virus free seed is essential for disease control. Current seed certification programmes rely on the visual assessment of seed lots tested in greenhouses. These methods are laborious and time consuming, require large areas of space and frequently fail to detect latent infections. The direct ELISA technique using raised antiserum was adapted for the detection of BCMV in seed lots (Appendix III). The antiserum, although not having an exceptionally high titre was shown by various methods to have a sufficiently high titre for use in the ELISA test and limited cross reactivity with host material.

Different serological tests were used to determine the titre of the antiserum raised in rabbit against the purified isolate, considered to be BCMV.

The microprecipitin test (Figure 16) was only used to give an indication of a rising titre after the immunization schedule. As soon as the titre was stable, the rabbit was bled by cardiac puncture. The test was advantageous as only minute quantities of reagents had to be used. Virus purity however, had to be extremely high so that precipitates could be clearly seen and not obscured by debris. The highest titre recorded was 1/256 as when the test was repeated after a further week, the titre had not increased appreciably. The rabbit was bled and the final titre was determined using the tube precipitin test (Figure 17). The test may have required greater volumes of reagents but precipitates were easier to detect. The titre was 1/512 using purified virus at a constant optimum concentration of 1/8. A bulky, flocculent precipitate, characteristic of eleongated virus particles, was produced after the overnight incubation at 4°C.

A parallel test was performed with purified, uninfected material. A very slight hazyness, indicating a low degree of cross reaction, was observed but only when high antibody concentrations were used.

As serum titre was low when determined by conventional methods, a more sensitive method, namely passive haemagglutination (Figure 18) was attempted. Virus could be detected in purified and partially purified sap. The titre varied between 1/512 and 1/1024 depending on the purity of the virus sample. In spite of having the potential for increased sensitivity, the results obtained were similar to those of the microprecipitin and tube precipitin tests.

The titre of the raised antiserum was not extremely high but it should be pointed out that there are difficulties when preparing virus preparations for antiserum production. Indeed BCMV itself is documented as being an extremely poor immunogen and the highest titre ever recorded has only been 1/2048 (Bos, 1971). It is difficult to obtain pure samples of any flexuous particle as host material tends to aggregate with virus during purification. For this reason, non specific host reactions frequently occur in serological tests. In the current study, little cross-reactivity was measured. To avoid <u>any</u> cross reactivity, serum can be cross absorbed to host fractions to remove anti-host-antibodies but this is not always practical, especially if serum is in short supply.

Double diffusion techniques were of little use as indicators of antibody titre. The titre was found to be much lower, 1/32, than obtained by other methods. Virus had to be disrupted using SDS before migration occured (Figure 19(a)) and it is likely that the properties of the disrupted virus protein differed from complete virus. Indeed, Moghal and Franki (1976) have stated that as

dissociated proteins are poorer immunogens, if reacted to serum to intact particles, the titre should be high to cause a reaction. It has also been suggested by Uyemoto et al. (1972) that better results can be obtained when antiserum is incorportated into the plates. This obviously depends on the availability of serum. In the double diffusion tests in the current study, strong precipitin lines formed between isolates 247, 7 (Potchefstroom) and 1 (Witkop) using raised antiserum. A weak reaction occured with healthy plant sap (Figure 19(a)).

The ELISA technique proposed by Clark and Adams (1977) was followed except for the use of a more concentrated gluteraldehyde solution in the coupling procedure, which would not have affected the enzyme (Avrameas and Ternyck, 1971). Colour intensity of the reaction was not read spectrophotometrically as there were no facilities, hence reagent concentrations had to be chosen to allow the difference between positive and negative (same as control wells) reactions to be readily assessed by visual means.

In the study, individual seed lots were also inoculated onto Phaseolus vulgaris var. "Bataaf" a variety highly susceptible to BCMV infections. A good correlation was obtained with the results of the ELISA technique (Table 13). Seed lots were tested not only for the presence, but also the distribution pattern of virus within the seed. The results suggest that BCMV can be readily detected in seedcoats and embryos yet only occasionally in cotyledons (Table 13). This proposed distribution pattern of BCMV in seed seems to correlate well with results published by Ephrain et al., (1974) and provides further evidence for the conclusion that the isolate studied is indeed BCMV. Their assay data also suggests that BCMV is internally borne, primarily in embryos and cotyledons and that low levels of infectivity are associated with

seedcoats which can be readily eliminated by surface decontamination of seeds. Soaking of all seeds tested probably accounted for the lack of detectable virus in seedcoats in the current study (Table 13). It has been suggested that BCMV in seedcoats becomes inactivated as the seed matures and the coat becomes dessicated and hence it is unlikely that this component is a real source of infection (Hoch and Provvidenti, 1978).

Ultrastructurally, the distribution pattern of BCMV in infected seed has also been investigated by Hoch and Provvidenti (1978). Virus aggregates and inclusions were observed in tissues of the embryonic axis and cotyledons which supports the experimental results recorded in the current study. Hence it appears that the successful transmission of BCMV is ensured due to the presence, and hence the protection, of infective particles within the actual seed component. For other bean viruses, such as southern bean mosaic virus, seed transmission is not as successful as the virus, contained only in seed coats, becomes inactivated with the maturation and storage of seeds.

Transmission of virus within seed further underlines the necessity for an efficient seed screening programme to detect viruses such as BCMV. The results of the current study indicate the potential of the ELISA for further use in the development and introduction of a seed screening programme for seed lots that are imported into the country. For such a programme to be established serum with an even greater titre than obtained in the current study would be desirable. Such serum, after cross adsorption with healthy sap would be able to detect even the lowest concentrations of virus in seed or seed components after germination, as was performed in this study.

Summary

Although 11 samples produced reactions on indicator plants, only 8 virus isolates were purified from the infected material collected in the Transvaal region. Most of the isolates appeared to be the same virus as they showed similar reactions on indicator plants, had a similar morphology and could be purified using the same methods. One isolate was completely different. It was a spherical particle, tentatively identified as TNV on the basis of host inoculations, limited biological properties and morphology. The virus was not subjected to further investigations. All other samples yielded flexuous particles of a similar size.

Sample 247 was selected as representative of the flexuous forms and as it was known to be seed borne, it could be used to test the ability of an ELISA procedure to detect virus in seed. The virus was purified and characterized using several techniques. The identity of this isolate (and 3 other samples) was confirmed to be BCMV using immunoelectronmicroscopy and double diffusion techniques. Recently, the isolate was also characterized as BCMV at Wageningen, Netherlands (Vermeulen, pers. comm., 1982).

BCMV is a well documented pathogen of legumes worldwide and has been suggested as existing in some of the major bean producing areas of South Africa. Identification of the virus has been based on symptomatology only and the virus as such has not been characterized previously in South Africa.

Results of differential host range studies, biological properties, particle morphology, capsid protein molecular weight, strandedness and molecular weight of RNA all compared favourably with the available data for BCMV.

Little is known about the strains of BCMV that occur in South Africa. Host reactions on varieties of *Phaseolus vulgaris* suggest that isolate 247 may be a new strain although a mixture of strains, present in the infected material could account for the results obtained.

The direct ELISA technique was used to detect virus in seed and results suggest a possible distribution pattern for the virus in infected seed. Recognition of the importance of seed transmission should promote the development of a reliable screening programme to detect BCMV in seed.

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Appendix I

Buffers for Purification

Method 2.3.1. (Whitlock, Price)

For homogenization:	0,1 M TRIS buffer, pH adjusted to 9,0 with thioglycolic acid (TGA).
For resuspension of sediments and preparation of gradients	0,1 M TRIS buffer, pH adjusted to 9,0 with hydrochloric acid (HCl).
Organic solvents:	Equal volumes of carbon tetrachloride and diethyl ether or chloroform.

Buffers and organic solvents are stored at 4°C.

Method 2.3.2. (Moat, 1976)

For homogenization:	M/20 (0,05 M) phosphate buffer, pH 7,6 0,1% TGA Butanol
For resuspension:	M/30 phosphate buffer
Phosphate Buffer:	M/2 (0,5 M) pH 7,6
$Na_2HPO_4.2H_2O$	8,9 g/100 mls H_2O
KH_2PO_4	6,8 g/100 mls H_2O

Mix 9 mls of KH_2PO_4 solution with 91 mls of $Na_2HPO_4.2H_2O$ solution. Adjust the pH to 7,6 with 2 N HCl.

Method 2.3.3. (Whitlock, 1979)

For homogenization:	Disodium hydrogen phosphate buffer 0,2 M ascorbic acid 0,1 M Butanol:Chloroform mixture 1:1
For resuspension:	0,03 M phosphate buffer pH 7,6

Method 2.3.4. (Jafarpour et al., 1979)

For homogenization:	0,05 M potassium phosphate buffer, pH 7,2 containing 0,5 M urea, 1% Na_2SO_3, 0,5 percent 2-mercapto-ethanol and 0,05 M EDTA.
	Chloroform
For resuspension and formation of gradients	0,025 M potassium phosphate buffer pH 7,2 containing 1 M Urea and 0,1% 2-mercaptoethanol.

Appendix II

Transmission Electron Microscopy

1. Coating of grids

a) Materials: 2% formvar stock solution
Chloroform

b) Methods: The stock formvar solution was diluted ten times. A cleaned slide was placed upright in the formvar for a few seconds. Once coated the slide was removed and dried upright on clear tissue paper. The slide was breathed on to detect the position of the film. A sharp blade was used to outline the edge of the film and this was floated off onto distilled water. The film was used to coat several 200 mesh copper grids. These were dried on filter paper, carbon coated, and stored in a petri dish until required.

2. Preparation and staining of thin sections

The following technique for the preparation of whole plant tissue was employed:

a) Materials:

1. Stock sodium cacodylate buffer, pH 7,2	21,4 g/litre gd H_2O
2. 25% stock gluteraldehyde solution	dilute 1:4 with sodium cacodylate buffer
3. 4% osmium tetroxide stock solution	dilute 1:4 with sodium cacodylate buffer
4. 0,5% aqueous uranyl acetate	
5. Alcohol	a 30% to 100% dilution series for dehydration is used.

Whole tissue was immersed in gluteraldehyde solution and cut into 1 cm x 1 cm pieces with a clean blade. The material was further sectioned into 0,5 - 1,0 mm cubes and transferred to fresh, buffered gluteraldehyde solution in a polytop vial. The material was left to fix for 40 minutes then rinsed three times in 0,1 M sodium cacodylate buffer for 30 minutes each time. Leaf material was post fixed in a mixture of equal volumes of 1% osmium tetroxide and 0,5 % aqueous uranyl acetate for 2 hours then rinsed times with buffer for 6 minutes each time.

Material was dehydrated using the ethanol series indicated below:

30 %	10 minutes
50 %	10 minutes
70 %	10 minutes
90 %	20 minutes
10 %	20 minutes
100 %	20 minutes

Spurr's Resin (Spurr, 1969).

a) Materials:

The standard embedding medium is formulated as follows:

ERL	5 grams
DER	3 grams
NSA	13 grams
S-1	0,2 grams (add just prior to use)

b) Methods:

All resin kit components were mixed thoroughly together. Dehydrated material was then subjected to the following procedure:

Propylene oxide	20 minutes
3:1 P.O./Spurr's	20 minutes
1:1 P.O./Spurr's	20 minutes
1:3 P.O./Spurr's	overnight
pure Spurr's	at least 3 hours

Plastic boats were 1/2 filled with pure Spurr's resin. A small piece of processed material was placed at the tip of each block and orientated into position. Specimens were polymerised for 2 hours at 80°C and exactly 8 hours at 70°C.

Appendix III

Serological procedures

1. Extraction of IgG from whole serum (Hudson and Hay, 1980).

a) Materials: 0,4% aqueous Rivanol
Saturated KBr 100g/100 ml gd H_2O
5 M saturated $(NH_4)_2SO_4$ 76 g/100 ml gd H_2O
Saline 0,877 g/100 ml H_2)
0,1 % NaOH

b) Methods:

The pH of rabbit serum was adjusted to 8,5 with 0,1 % NaOH. The serum was diluted with saline by addition of half of the original volume. To another tube, Rivanol (3,5 times the original volume of serum) was added. The Rivanol was gradually pipetted into the serum. The sample was mixed after each addition on a vortex mixer. The sample remained at room temperature for 10 minutes before centrifugation at 10,000 RPM for 10 minutes. To the decanted supernatent fluid, saturated KBr was added dropwise to approximately half of the volume. The fluffy yellow rivanol-bromide precipitate was removed by centrifugation at 15,000 RPM for 20 minutes. To the resultant supernatent fluid 2/3 of the volume of saturated ammonium sulphate was added. The sample was left overnight at 4°C to complete IgG precipitation. IgG was precipitated by centrifuation at 10,000 RPM for 15 minutes. The pellet was redissolved in 15 ml of saline and 10 ml of $(NH_4)_2SO_4$ was again added. The process of centrifugation and precipitation was repeated twice. The final IgG precipitate was dissolved in 1-2 ml of saline and dialysed against several changes of gd H_2O at 4°C for three days. The concentration of IgG in the final preparation was

determined as follows:

an O.D. of 1 at 280 = 0,74 mg/ml IgG

The concentration of IgG was adjusted with saline until an O.D. of 1,40 at 280 nm was obtained. IgG was concentrated and preserved by freezedrying. The purity and presence of IgG was detected by PAGE on 7% gels and immunoelectropheresis on agarose gels.

2. The ELISA technique (Voller et al, 1979 ; Clark and Adams, 1977

a) Materials:

i. The following buffers were used:

a) Coating buffer:

0,05 M sodium carbonate at pH 9,6

Na_2CO_3	1,5 g	
$NaHCO_3$	2,93 g	
NaN_3	0,2 g	Make up to 1 litre and adjust the pH to 9,6

b) Substrate buffer:

10 % diethanolamine adjusted to pH 9,8 with HCl

diethanolamine	48,5 mls	
gd H_2O	400,0 mls	
NaN_3	0,1 g	Adjust the pH to 9,8 with HCl and make up to 500 ml

c) PBS:

1 tablet / 100 ml of gd H_2O

d) PBS-Tween 20:

PBS, pH 7,4 with 0,05 % Tween 20

e) 3 M NaOH:

120 g NaOH / 1 litre gd H_2O

All buffers for stopping the hydrolysis of substrate contained 0,02 % sodium azide as preservative.

ii. Enzyme:
The enzyme alkaline phosphatase was used

iii. Substrate:
2-nitrophenyl phosphate was used at 1,0 mg / ml concentration in 1 ml of substrate buffer

iv. Polystyrene microtitre plates

v. Test samples:
Tests were made with purified virus samples, sap extracts, seeds and seed components.

b) Methods:

1. Preparation of the coating immunoglobulin

One mg of purified freeze dried IgG was dissolved in 1 ml of coating buffer. The stock solution was serially diluted for the grid titration to 10 µg/ml 1 µg/ml and 0,1 µg/ml in coating buffer. Samples were stored in silicon treated glass vials at 4°C until used.

2. Preparation of the enzyme-antibody conjugate

One mg of purified freeze dried IgG was dissolved in 2 mg of alkaline phosphatase. Approximately 0,5 mls of saline was also added. The mixture was dialysed extensively against several changes of PBS overnight at 4°C. 10 µl of 25% stock gluteraldehyde solution was added and the conjugate was then concentrated 10 times using a 'minicon' before being stored in silicon treated tubes at 4°C in the presence of 1% BSA for protection. The conjugate was diluted for use in the grid titration to 1/22, 1/800 and 1/3200 in PBS-Tween 20.

3. Preparation of antigen samples

Virus was purified from systemically infected P. vulgaris var. "Double Dutch Princess", plants using the standard procedures proposed by Gafarpour et al. (1979). The final step was differential centrifugation. Purified preparations were diluted to 1/10, 1/100 and 1/1000 in PBS-Tween 20 for use in the grid titration. Sap samples were prepared by grinding infected leaves without buffer in a mortar and pestle. Sap was used concentrated or diluted to 1/10, 1/100 and 1/1000 in PBS-Tween 20.

Seed samples were prepared by soaking about 50 seeds of each seed lot in separate petri dishes for 48 hours. Twenty germinating seeds were then selected from each batch. Each seed was carefully dissected into the following components: embryo, seed coat, cotyledon and remaining whole seeds. Individual components were ground in about 2 to 5 mls of PBS-Tween 20 in a mortar and pestle. 200 µl of each solution was placed in individual wells previously coated with IgG at the optimum concentration for colour reaction determined by the grid titration. They were incubated overnight at 6°C, washed then coated with conjugate. Addition of substrate and sodium hydroxide were carried out as before for the ELISA. The results were assessed visually based on the intensity of the colour reaction.

The seed samples tested were the following:

1. APC 84 C7
2. Various Lines E
3. PC 97 69 E A
4. Yellow haricot
5. DP3
6. Pinto U1 114 A
7. Bonus Colombian BCMV
8. DP 8 & 7
9. PC 10 D
10. PC 9826 1 7*/79
11. APC 97E 25 P1
12. APC 97E 31 P1
13. BPC 97 34 E1
14. APC 84C 8
15. APC 94 27 P2
16. PC 97 21 E1
17. PC 84 C1 A
18. APC 9417 H2
19. A PRETO 145

The origins of the seed samples are as follows:

A. From a yield trial at Potchefstroom
B. From the breeders' nursery at Potchefstroom
C. From the breeders' nursery at Ermelo
D. From a yield trial at Potchefstroom with seed from various sources (variety P. vulgaris "Bonus")
E. Seed from plants with virus like symptoms seen in Potchefstroom 1980/1981 - unlikely to be bean common mosaic virus

The crosses of the various samples are as follows:

PC 84 : (Burmea x Great Northern) x (Seafarer x Coffee)
PC 94 : (Seaway x WDP.SPS 25) x NEP II
PC 97 : (Seafarer x Coffee) x NEP II
PC 98 : (Michelite x Great Northern) x NEP II

The lines from these crosses were segregates i.e. selected as single plants in the F_4 generation.

Author Cowper B A
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