

A STUDY OF FILAMENTOUS VIRUSES  
IN MAIZE AND SMALLGRAINS

BY

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## ABBREVIATIONS

|               |                                         |
|---------------|-----------------------------------------|
| Ag            | Antigen                                 |
| Ab            | Antibody                                |
| BaYMV         | Barley yellow mosaic virus              |
| BMV           | Brome mosaic virus                      |
| BPB           | Bromophenol Blue                        |
| BSA           | Bovine serum albumin                    |
| CMV           | Cucumber mosaic virus                   |
| cv.           | Cultivar                                |
| EDTA          | Ethylene diamine tetra-acetic acid      |
| ELISA         | Enzyme linked immunosorbent assay       |
| GAR-conjugate | Goat anti-rabbit conjugate              |
| GGMV          | Guinea grass mosaic virus               |
| gdw           | 2 x glass distilled water               |
| IEB assay     | Immuno electroblot assay                |
| IgG           | Gamma globulin fraction of serum        |
| ISEM          | Immunosorbent electron microscopy       |
| Kd            | Kilodaltons                             |
| LMV           | Lettuce mosaic virus                    |
| MDMV          | Maize dwarf mosaic virus                |
| MDMV-ST       | Seed transmitted MDMV                   |
| MW            | Molecular weight                        |
| PAGE          | Polyacrylamide gel electrophoresis      |
| PBS           | Phosphate buffered saline               |
| PEG           | Polyethylene glycol                     |
| Rate Z        | Rate zonal centrifugation               |
| SDS           | Sodium dodecyl sulphate                 |
| SCMV          | Sugarcane mosaic virus                  |
| TEMED         | N, N, N', N' tetramethylethylenediamine |
| TMV           | Tobacco mosaic virus                    |
| Tris          | Tris hydroxymethylaminomethane          |
| ss-RNA        | single stranded ribonucleic acid        |
| 170S          | Sedimentation coefficient in Svedbergs  |
| WSMV          | Wheat streak mosaic virus               |
| ZE            | Zone electrophoresis                    |
| u             | $\mu$ as in ug, ul, etc.                |
| UV            | Ultraviolet                             |

## LIST OF ANTISERA

| <u>Antiserum</u>                                | <u>Antigen</u>              |
|-------------------------------------------------|-----------------------------|
| 1. <u>anti MDMV/SCMV sera</u>                   |                             |
| anti-MDMV-A (U.S.)                              | see Section IVA.9(a) (iii)  |
| anti-MDMV-B (U.S.)                              | "                           |
| anti-MDMV-Bar-1                                 | see Section IVa.6           |
| anti-MDMV-Bar-2                                 | "                           |
| anti-MDMV-Kru-1                                 | "                           |
| anti-MDMV-Kru-2                                 | "                           |
| anti-MDMV-Win-1                                 | "                           |
| anti-MDMV-Win-2                                 | "                           |
| anti-MDMV-ST                                    | "                           |
| anti-SCMV 4975-1                                | "                           |
| anti-SCMV 4975-2                                | "                           |
| anti-SCMV 376 (UCT antiserum collection)        | Old SCMV isolates (UCT)     |
| anti-SCMV-Martin-1 (UCT antiserum collection)   | "                           |
| anti-SCMV-Martin-2 (UCT antiserum collection)   | "                           |
| 2. <u>Other</u>                                 |                             |
| anti-maize Fraction 1                           | maize-fraction-1<br>protein |
| anti-Abrahamskraal-1 (UCT antiserum collection) | Abrahamskraal<br>isolate    |
| anti-Abrahamskraal-2 (UCT antiserum collection) | "                           |
| anti-BaYMV (UCT antiserum collection)           | BaYMV (crude<br>extract)    |
| anti-CMV-1 (UCT antiserum collection)           | CMV-Tob                     |
| anti-CMV-2 (UCT antiserum collection)           | CMV-Is                      |
| anti-TMV (UCT antiserum collection)             | TMV                         |
| anti-WSMV (M.K. Brakke, U.S.)                   | WSMV                        |

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CHAPTER I  
INTRODUCTION

The occurrence of maize dwarf mosaic virus (MDMV) in field grown maize was investigated. For this purpose, maize showing mosaic symptoms was collected from different maize growing areas in South Africa by Prof. M.B. von Wechmar. These samples from Transvaal, Orange Free State and Natal were then investigated for the presence of MDMV and possible strains of this virus. Three virus isolates were purified and partially characterised. These isolates were serologically compared together with a fourth isolate SCMV 4975, obtained from the U.S., to establish strain relationships.

Small grains and maize are often grown in the same regions. Several species of brome grass are known to be susceptible to both MDMV strains A and B (Rosenkranz, 1980). MDMV has also been reported to occur occasionally as symptomless infections in barley (Gates, 1983). In view of these facts as well as the presence of filamentous viruses found in field collected brome grass (Bromus catharticus), barley (Hordeum vulgare) and Johnsongrass (Sorghum halepense), it was of interest to test whether maize viruses could infect these hosts. These isolates were therefore serologically compared to the MDMV isolates. The possibility that one of these could be WSMV (poty-like virus) was investigated. WSMV and MDMV are known to occur in double infections in maize (Ford and

Lambe, 1967; Moline, 1973).

During the course of the investigation, it was found that a filamentous virus was transmitted in a small proportion of maize seeds. This phenomenon was further investigated in order to establish the extent of seed transmission and also to characterize some properties of this isolate. Seed transmission was expressed in two ways: as maize symptoms in some plants and symptomless infections in others. It is possible that there was a mixture of two filamentous viruses in maize.

An isometric virus was also found to be present in maize and was not fully investigated here. Miss E Knox (1983 Hons. project, U.C.T.) had shown by serological tests that cucumber mosaic virus was present in the seed of two maize lines, with one maize line containing brome mosaic virus antigen as well.

CHAPTER II  
LITERATURE REVIEW

A. INTRODUCTION

The first maize virus was found in the U.S.A. in the 1920's when Brandes discovered a mosaic disease of maize found near sugarcane fields (Gordon et al. 1981). This virus was identified as sugarcane mosaic virus (SCMV). From the beginning of the 1960's maize viruses became more important in the U.S.A., when major epiphytotics occurred (Shepherd 1965, Louie et al. 1974, Damsteegt 1976). In 1965 Williams and Alexander described the virus associated with the maize dwarf disease as maize dwarf mosaic virus (MDMV) and since then MDMV has been found in most of the maize growing areas in the U.S.

In South Africa, Storey (1929) reported the presence of a virus which he first detected in 1924 near Pretoria in Sorghum arundinaceum Stapf., a wild grass. The virus caused a mosaic disease on maize and sorghum and was not distinguishable from SCMV by symptoms, but it did not infect sugarcane. Later, in 1967, Von Wechmar and Hahn reported a separate strain of SCMV infecting sorghum, on the basis of serological tests. Erasmus (1979, Hons. project, U.C.T.) compared a sorghum and two sugarcane isolates using the indirect ELISA method, and found that

all three were closely related serologically to an SCMV strain from sugarcane (von Wechmar and Hahn, 1967).

Erasmus (1979, Hons. project U.C.T.) was the first to use ELISA for serological comparison of SCMV isolates in South Africa.

SCMV and MDMV are considered strains of the same virus (Pirone, 1972). Both viruses infect similar host ranges restricted to the Gramineae family. SCMV and MDMV are members of the Potyviridae group. MDMV and SCMV occur worldwide, in the States, Europe, Asia and Africa (Gordon et al. 1981). Shepherd (1965) has reported that MDMV and SCMV are serologically closely related. Some authors, e.g. Ford and Hill (1976) regard MDMV as a synonym for SCMV. This opinion is not shared by all. At least 13 different strains of SCMV have been differentiated by symptomatology on sugarcane varieties (Pirone, 1972). Six different strains of MDMV, ranging from MDMV-A to -F, have been reported by Louie and Knoke (1975). Mackenzie et al. 1966, reported the strain of MDMV that did not infect Johnsongrass, i.e. MDMV-B. Nearly all the SCMV and MDMV strains are serologically related (Tosic and Ford, 1974; Gordon et al., 1981).

For the purposes of this thesis, the review will concentrate on MDMV.

## B. HOST RANGE AND SYMPTOMS

A broad generalization of symptoms produced by MDMV in the hosts it infects is given. Many hosts show a mosaic symptom and in some cases, e.g. maize (Zea mays L.), MDMV causes dwarfing (Williams and Alexander, 1965; Seghal and Jean, 1968; Tosic and Ford, 1972). Some varieties of sorghum show necrosis and leaf reddening when infected with MDMV (Pirone, 1972; Martin and Hackerott, 1982). Temperature also affects the symptoms produced as shown by Jarjees and Uyemoto (1983). Two sorghum inbreds when infected with MDMV-A showed mosaic symptoms, whereas at lower temperatures one of the inbreds was reported to show red leaf disease (i.e. tissue necrosis). No suitable local lesion hosts have been reported for MDMV, although some workers, e.g. Louie and Knoke (1975) have used specific maize inbreds which form local lesions when infected with MDMV. Maize, a systemic host, has been used as an assay host by most workers (Shepherd and Holdeman, 1965; Seghal and Jean, 1968; Williams et al. 1968; Tosic and Ford, 1972, 1974; Louie and Knoke, 1975; Rosenkranz, 1978, 1980, 1983; Panayatou, 1981 and Gates, 1983). The principle cultivated crops infected by MDMV are maize, sugarcane and sorghum (Pirone, 1972). In fact MDMV has a very wide host range which, however, is limited to the Gramineae family. The hosts of MDMV are found in all six subfamilies of the Gramineae (Rosenkranz, 1983) and over three hundred grass species are known to be hosts of MDMV (Rosenkranz, 1978,

1980, 1983; Tasic and Ford, 1972). Dicotyledonous plants have been tested for susceptibility to SCMV but all reports have been negative (Shepherd, 1965; Williams and Alexander, 1965; Paulsen, (1966) cited in Ford and Tasic (1972). The greatest number of susceptible hosts in the Gramineae family were found in the subfamily Panicoideae as reported by Rosenkranz (1983). He reported that a large number of natural grasses had latent infections of MDMV. The latent infections were detected by the presence of symptoms on maize that had been inoculated with the infected grass samples. The highest percentage of species in the various subfamilies showing latent infections were found in the subfamily Festucoideae.

Table I shows a summary of a selected broad host range of the major MDMV and SCMV strains as reported by various authors.

Table 1 : A selected host range of MDMV and SCMV

| Host                                                                                | MDMV-Strain-A                             | MDMV-Strain-B        | MDMV                                          | SCMV-Strain-B      | SCMV               |
|-------------------------------------------------------------------------------------|-------------------------------------------|----------------------|-----------------------------------------------|--------------------|--------------------|
| Sugarcane<br>( <u>Saccharum officinarum</u> L.)                                     | - <sup>6</sup><br>+ <sup>3</sup>          | - <sup>3</sup>       | + <sup>1</sup>                                | + <sup>3</sup>     | + <sup>3,1</sup>   |
| Maize*<br>( <u>Zea Mays</u> L.)                                                     | + <sup>1,4,5,6,7</sup>                    | + <sup>1,4,7</sup>   | + <sup>2</sup>                                | + <sup>4</sup>     |                    |
| Sorghum<br>( <u>Sorghum vulgare</u> Pers.<br>= <u>Sorghum bicolor</u> (L.) Moensch) | + <sup>6,7</sup><br>+ <sup>1,5</sup> \$   | + <sup>4,5</sup>     | + <sup>7</sup>                                | + <sup>1,3,7</sup> |                    |
| Johnsongrass<br>( <u>Sorghum halepense</u> (L.) Pers)                               | + <sup>3,4,5,7,</sup><br>+ <sup>1,6</sup> | - <sup>1,3,4,7</sup> | + <sup>1</sup>                                | - <sup>3,4</sup>   | - <sup>1,3,7</sup> |
| Wheat<br>( <u>Triticum aestivum</u> L.)                                             | - <sup>1,6</sup>                          | - <sup>1</sup>       | - <sup>2</sup>                                | + <sup>3</sup>     | - <sup>1</sup>     |
| Barley<br>( <u>Hordeum vulgare</u> L.)                                              | - <sup>6,7</sup>                          | - <sup>7</sup>       | - <sup>1</sup><br>+ <sup>S<sup>2</sup>@</sup> | + <sup>3</sup>     | - <sup>1,7</sup>   |
| Oats<br>( <u>Avena sativa</u> L.)                                                   | - <sup>1</sup>                            | - <sup>1</sup>       | + <sup>S<sup>2</sup></sup>                    |                    | - <sup>1</sup>     |

Numbers refer to sources: 1 = Ford and Tomic (1972); 2 = Gates (1983); 3 = Pirone (1972);  
 4 = Rosenkranz (1980); 5 = Seghal and Jean (1968); 6 = Shepherd (1965);  
 7 = Tomic and Ford (1972).

+ = inoculated virus shows symptoms in these hosts  
 - = inoculated virus does not show any symptoms in these hosts  
 \$n = virus occurs naturally in this host  
 @s = symptomless host of virus

\* A number of sweetcorn varieties of maize were used by various authors, including Burpee Golden Cross Bantam, Golden Giant, Seneca Chief and Goldein Bantam. The sweet corn was used as an assay host for back-inoculation from symptomless hosts.

A wide range of Bromus species were tested for MDMV susceptibility by various workers. Six of the Bromus species were symptomless hosts, namely B. autelicus (Ford and Tasic, 1972), B. oxydon, B. rubens, B. scoparius, B. japonicus and B. tectorum (Rosenkranz, 1983). Table 2 gives a summary of various Bromus species susceptible to MDMV strains A and B, SCMV-B and the Johnsongrass strain of SCMV (SCMV-Jg).

Special reference is made to the Bromus species as a filamentous virus isolated from Bromus catharticus was investigated in this project.

Table 2 : Various Bromus species susceptible to MDMV and SCMV strains

| Species                             | MDMV-A                              | MDMV-B                           | MDMV            | SCMV-B          | SCMV            | SCMV-Jg        |
|-------------------------------------|-------------------------------------|----------------------------------|-----------------|-----------------|-----------------|----------------|
| <u>B. arvensis</u> L.               | + <sup>1,7</sup>                    | + <sup>1,7</sup>                 |                 |                 | + <sup>7</sup>  |                |
| <u>B. auleticus</u> Trinex Griseb.  | - <sup>7</sup>                      | - <sup>7</sup>                   | + <sup>S1</sup> |                 | - <sup>7</sup>  |                |
| <u>B. brizaeiformis</u> Fisch + Mey | + <sup>2</sup>                      | + <sup>2</sup>                   |                 | + <sup>2</sup>  |                 |                |
| <u>B. catharticus</u> Vahl.         | - <sup>1,3,5</sup>                  | - <sup>3</sup>                   | - <sup>1</sup>  | - <sup>3</sup>  |                 |                |
| <u>B. commutatus</u> Schrad.        | - <sup>1,4</sup>                    | - <sup>4</sup>                   | + <sup>1</sup>  | - <sup>4</sup>  |                 |                |
| <u>B. danthoniae</u> (Desf.) Trin   | + <sup>1,5,7</sup>                  | + <sup>7</sup>                   |                 |                 | + <sup>7</sup>  |                |
| <u>B. erectus</u> Huds.             | - <sup>1</sup>                      |                                  |                 |                 |                 |                |
| <u>B. inermis</u> Leyss.            | - <sup>1</sup>                      | - <sup>1</sup>                   |                 |                 | - <sup>1</sup>  |                |
| <u>B. japonicus</u> Thunb.          | + <sup>S4x</sup><br>+ <sup>7</sup>  | + <sup>S</sup><br>+ <sup>7</sup> | + <sup>1</sup>  | + <sup>S4</sup> | + <sup>7</sup>  |                |
| <u>B. lanceolatus</u> Roth.         | + <sup>1,5</sup><br>+ <sup>sn</sup> | - <sup>7</sup>                   |                 |                 | + <sup>7</sup>  |                |
| <u>B. macrostachys</u> Desf.        | + <sup>1,5,7</sup>                  | + <sup>7</sup>                   |                 | + <sup>7</sup>  |                 |                |
| <u>B. marginatus</u> Nees.          |                                     |                                  | - <sup>1</sup>  |                 |                 |                |
| <u>B. mollis</u> L.                 | + <sup>6</sup><br>- <sup>7</sup>    | + <sup>7</sup>                   | + <sup>1</sup>  |                 |                 | + <sup>7</sup> |
| <u>B. oxydon</u> Schrenk.           | + <sup>S7</sup>                     | + <sup>S7</sup>                  | + <sup>S1</sup> |                 | + <sup>S7</sup> |                |
| <u>B. purgans</u> L.                |                                     |                                  | - <sup>1</sup>  |                 |                 |                |
| <u>B. racemosus</u> Huds.           | - <sup>1</sup>                      |                                  |                 |                 |                 |                |

|                               |                    |                |                                      |
|-------------------------------|--------------------|----------------|--------------------------------------|
| <u>B. rigidus</u> Roth.       | + <sup>1,5,7</sup> | + <sup>7</sup> | + <sup>7</sup>                       |
| <u>B. rubens</u> L.           | - <sup>7</sup>     | + <sup>7</sup> | + <sup>7</sup>                       |
| <u>B. scoparius</u> L.        | + <sup>1,5</sup>   | - <sup>7</sup> | + <sup>7</sup> (SCMV-H) <sup>7</sup> |
| <u>B. secalinus</u> L.        | - <sup>1</sup>     | + <sup>3</sup> |                                      |
| <u>B. sitchensis</u> Trin.    | + <sup>3</sup>     | - <sup>1</sup> |                                      |
| <u>B. squarrosus</u> L.       | - <sup>1</sup>     | + <sup>2</sup> |                                      |
| <u>B. sterilus</u> L.         | + <sup>2</sup>     |                |                                      |
| <u>B. tectorum</u> L.         | - <sup>1</sup>     | + <sup>4</sup> | + <sup>7</sup>                       |
| <u>B. tomentellus</u>         | + <sup>4</sup>     | + <sup>7</sup> | + <sup>7</sup>                       |
| <u>B. trinii</u> Desv.        | + <sup>2</sup>     | + <sup>2</sup> |                                      |
| <u>B. variegatus</u> Marsche. | - <sup>1</sup>     |                |                                      |

Numbers refer to sources: 1 = Ford and Tosic, (1972); 2 = Rosenkranz (1978); 3 = Rosenkranz (1980); 4 = Rosenkranz (1983); 5 = Seghal and Jean (1968); 6 = Shepherd (1965); 7 = Tosic and Ford (1972).

- + = inoculated virus shows symptoms in these hosts.
- = inoculated virus does not show any symptoms in these hosts.
- x s = symptomless host of virus.
- 0 sn = host shows symptoms but negative results on reinoculation.

### C. PHYSICAL CHARACTERISTICS OF MDMV

A summary of the physical and biological characteristics of MDMV is given in Table 3.

Table 3 : The physical and biological characteristics of MDMV

#### A. Physical characteristics

|                                              |                                                                                                                                              |
|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|
| Morphology                                   | Flexuous rod shaped particle<br>750-755nm (Bancroft <u>et al.</u> 1966,<br>Shepherd, 1965) 11-13nm diameter                                  |
| Sedimentation<br>coefficient                 | 148-170S (Shepherd, 1965; Seghal,<br>1968; Bancroft <u>et al.</u> 1966; Jones<br>and Tolin, 1972; Langenberg, 1973;<br>Tosic and Ford, 1974) |
| Buoyant density in<br>CsCl                   | MDMV-A 1,3421g/cm <sup>3</sup> (Tosic and Ford, 1974)<br>MDMV-B 1,3427g/cm <sup>3</sup> ( " " " " )                                          |
| Extinction coefficient,<br>$E_{260}^{0,1\%}$ | 2,7 (Langenberg, 1973)                                                                                                                       |
| 260/280 Absorbance ratio                     | 1,17-1,26 (Seghal and Jean, 1968;<br>Langenberg, 1973)                                                                                       |
| <u>Protein subunit</u>                       |                                                                                                                                              |
| MW                                           | 36,5 x 10 <sup>3</sup> d(SDS-PAGE) (Hill <u>et al.</u> 1973)<br>28,5 x 10 <sup>3</sup> d(AA analyses) ( " " " " )                            |
| No. of amino acids                           | 264 (Hill <u>et al.</u> 1973)<br>290 (Hollings and Brunt, 1981(a))                                                                           |
| <u>Genome</u>                                |                                                                                                                                              |
| % RNA                                        | 6% (Hill <u>et al.</u> 1973)                                                                                                                 |
| MW                                           | 2,7 x 10 <sup>6</sup> d (Pring and Langenberg, 1972)                                                                                         |

Table 3 continued

B. Biological characteristics

|                                  |                                                                                                                                              |                             |
|----------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| Host range                       | Restricted to Gramineae                                                                                                                      |                             |
| Thermal inactivation point (TIP) | 54-58°                                                                                                                                       | )                           |
| Dilution end point (DEP)         | $10^{-2}$ - $10^{-5}$                                                                                                                        | )                           |
| Longevity in vitro (LIV)         | 1 - 2 days at room temp<br>3 - 5 days at 4°C                                                                                                 | )                           |
|                                  |                                                                                                                                              | ) Reviewed by Gingery, 1981 |
| Situation in cells               | Virus scattered in cytoplasm, occasionally in plasmodesmata.                                                                                 |                             |
|                                  | Induces cylindrical cytoplasmic inclusions (Krass and Ford, 1969)                                                                            |                             |
| Transmission                     | Mechanical<br>Non-persistent transmission by 20 aphid species (Aphid transmission reviewed by Knoke and Louie, 1981; Nault and Knoke, 1981). |                             |

#### D. STRAIN IDENTIFICATION

Numerous strains of MDMV and SCMV have been recorded (Mackenzie et al. 1966; Louie and Knoke, 1975; Zummo and Gordon, 1971; Abbot and Tippet, 1966; Koike and Gillaspie, 1976; Shukla and Gough, 1984). Various workers have serologically tested different strains to establish relationships (Snazelle et al. 1971; Totic et al. 1973; Totic and Ford, 1974). Whether these strains are different or whether they were different isolates recorded by different workers remains to be tested against several known standards by using sensitive and critical criteria, e.g. in ELISA, IEB and ISEM assays. Gordon (1984) and Gillaspie et al. (1984) have suggested that more stringent conditions and preferably back testing with known strains should be done to clearly characterize strains/isolates.

## E. VECTOR TRANSMISSION

MDMV is transmitted in a non-persistent manner by more than 20 different aphid species (Knoke and Louie, 1981). Nault and Knoke (1981) reviewed the transmission of MDMV by aphids. All the species do not have the same efficiency of transmission. Bancroft et al. 1966, when testing 10 aphid species found that Dactynotus sp was the most efficient vector whereas Rhopalosiphum padi sp (L) and R. maidis (Fitch) were the least efficient vectors. Myzus persicae which is known to transmit more than 70 viruses non-persistently (Matthews, 1981), was of intermediate efficiency as a vector. A fairly large amount of aphid transmission tests with strains of MDMV and different aphid species has been done by several workers :

Messieha (1967); Shaunak and Pitre (1973); Louie and Knoke (1975); Thongmeearkom et al. (1976); Straub (1982) and Berger et al. (1983). Short acquisition threshold periods of 10 - 30 seconds have been reported and retention periods of 15 - 20 minutes have been reported by Bancroft et al. (1966) and Messieha (1967) whereas longer retention periods of up to 240 minutes have been reported by Thongmeearkom et al. (1976) for MDMV-A retention by Myzus persicae. Johnsongrass is as effective a virus source as maize (Messieha, 1967). Various isolates and strains of MDMV are transmitted at different rates by aphids (Messieha, 1967; Louie and Knoke, 1975). Different biotypes of the same aphid species may also differ in

efficiency of transmission of MDMV. For instance, Berger et al. (1983) found that a new biotype of Schizaphis graminium (biotype E) was more efficient than biotype C as a vector of both MDMV-A and -B. An interesting feature of transmission of Potyviruses by aphids, is that a specific 'helper component' present in infected plants, is required for transmission (Govier and Kassanis, 1974; Pirone, 1981; Pirone and Thornbury, 1983 and Matthews, 1981). Tests for the presence of helper component in MDMV infected leaves have not yet been reported.

Aphid transmission is important in the secondary spread of virus. Straub (1982) found in New York that younger seedlings, i.e. maize seed planted later in the season, when large numbers of aphids were present, led to 100% infection of maize crop and yield reductions of 85%.

There have been reports of longer retention times by aphids carrying MDMV by Berger and Zeyen (1981). Aphids retained MDMV for 18 - 70 hours when they were gassed or placed at 7°C and not allowed to probe, to simulate their behaviour in air currents. Berger and Toler (1983b) also determined that MDMV was retained longer when the time of acquisition was increased. Berger and Zeyen (1981) have suggested that the rapid spread of MDMV in the U.S.A. may be due to the long range dispersal of MDMV by aphids carried in low level winds.

## F. YIELD LOSSES

Yield losses due to MDMV infection in maize crops have been recorded to range from 10 to 54% (Williams and Alexander, 1965; Gregory and Ayers, 1982; Kuhn and Smith, 1977; Gordon et al. 1981).

Various methods for controlling virus disease spread have been suggested, e.g. use of insecticides (Ferro and Mackenzie, 1980), application of carbofuran to soil to prevent further dissemination of virus by vectors (Rains and Christensen, 1983). But these methods would only prevent secondary spread of the virus.

A more effective way of control would be to breed maize lines for resistance to virus, e.g. as suggested by Scott and Findley, 1984 and Martin et al. 1984.

It has been shown that different maize lines have different responses to MDMV infection (Louie et al. 1976).

Breeding maize for resistance to MDMV is important in the light of the seed transmission of the virus.

## G. SEED TRANSMISSION

At least twelve Potyviruses are known to be transmitted through seed of infected plants, e.g. lettuce mosaic virus, soy bean mosaic virus, pea seed-borne mosaic virus (Brlansky and Derrick, 1979; Chen et al. 1982; Goodell and Hampton, 1983; and Hollings and Brunt, 1981 a).

In some cases a large proportion of seed carry virus, e.g. LMV was found in 3 - 29% of lettuce seed (Zink et al. 1956), whereas MDMV has been reported to be transmitted through a small proportion of maize seed. Shepherd and Holdeman (1965) reported MDMV transmission in 0,4% of maize seed, Hill et al. (1974) 0,2%, Totic and Sutic (1977) 0,008% and Williams et al. (1968)  $6,7 \times 10^{-5}\%$  of seed.

Since 1965, when Shepherd and Holdeman first reported that the Johnsongrass strain of SCMV was transmitted through seed, there have been a number of contradictory reports where a number of authors having tested for seed transmitted MDMV, obtained negative results. Bancroft et al. (1966) tested 900 maize seed from infected field grown plants and seed of Sorghum vulgare (wild cane) but did not detect any symptoms in seedlings grown from this seed. Ford and Hill (1976) could not detect seed transmission of MDMV-A or -B in Johnsongrass, Sorghum alnum, dent maize or in four sweetcorn cultivars, including cv.

'Golden Bantam'. Later in 1981 Ranayatou tested for the presence of seed-transmitted MDMV-A in 3 maize hybrids (12 500 seeds were examined). This was tested by examining plants for presence of symptoms and by mechanical inoculation onto indicator maize plants, but he obtained negative results. Markov (1980) in Bulgaria, showed that the maize mosaic virus (which is probably a virus related to MDMV) was found in immature seed but could not find virus in the mature maize seed.

In contrast to these reports, several authors reported a low percentage of MDMV transmitted through maize seed. For instance, Shepherd and Holdeman (1965) found MDMV in 0,4% of seed of field corn and sweetcorn based on the detection of symptoms. They obtained negative results when testing Johnsongrass and sorghum, but they reported that these results were not conclusive as single trials with small samples (500 and 900 seeds respectively) were used. Williams et al. (1968) reported a very low percentage ( $6,7 \times 10^{-5}\%$ ) of MDMV-A in maize seed, as only 2 out of 29 735 seedlings were positive. They had examined 477 inbred lines, inbred strains, crosses and hybrids of dent, sweetcorn and popcorn by growing seed obtained from plants in areas with a high occurrence of MDMV and testing for presence of symptoms. MDMV was detected in 0,2% of seed by symptom expression in one of 13 maize inbred lines examined by Hill et al (1974). The authors also reported

a 0,1% seed transmission of wheat streak mosaic virus (WSMV), which is the first report for the seed transmission of WSMV in maize. MDMV and WSMV were found to be transmitted together in a single maize seed. These latter results had been determined by microprecipitin tests of crude extracts of plants showing mosaic symptoms. The double infection of maize by MDMV and WSMV has been reported previously by How (1963). Hill et al. (1974) tested several maize inbred lines for the response to MDMV-B infection. Although all the maize lines were susceptible to MDMV-B, some plants were reported to have latent infections, and MDMV-B had been recovered from these plants with no symptoms. The fact that these latent infections do occur, shows that the practice of examining plants for symptoms without testing serologically, is not very reliable. Tomic and Sutic (1977) also reported a low percentage (0,008%) of MDMV-A transmission in maize seed. Von Wechmar and Chauhan (1984) found that SCMV (serologically related to MDMV-B) was transmitted through maize seed (von Wechmar and Chauhan (1985, submitted)). The seed transmitted virus was detected by symptom expression and by testing embryos of a small sample of maize seed by the sandwich ELISA test. They found that the rate of germination of maize seedlings was correlated with the presence of SCMV in the seed. They also found latent infections of SCMV in maize. In all earlier work von Wechmar and co-workers referred to SCMV (Von Wechmar, 1967; von Wechmar and

Hahn 1967, von Wechmar 1983 and von Wechmar and Chauhan 1984). A re-investigation of earlier isolates and antisera prepared to such isolates showed that isolates of maize and sugarcane were, in fact, of the MDMV-B type. Reference to these earlier isolates as SCMV could have led to a confusion of nomenclature. An attempt was made by von Wechmar to correct this to avoid a continuation of confusing nomenclature. In summary, virus isolated from maize or sorghum will in future be referred to as MDMV; viruses isolated from sugarcane and studied in sugarcane will be referred to as SCMV (von Wechmar and Chauhan, 1985, submitted).

## H. OTHER FILAMENTOUS VIRUSES

Filamentous viruses, other than MDMV, are known to infect maize and barley. Table 4 shows some characteristics of wheat streak mosaic virus (WSMV), barley yellow mosaic virus (BaYMV) and Guinea grass mosaic virus (GGMV).

WSMV was first reported as a mosaic disease of maize by Finley (1954) in the U.S.A. WSMV is not classified as a Potyvirus (Hollings and Brunt, (1981) although it has many characteristics similar to the Potyviruses. The latter are transmitted by aphids whereas WSMV is transmitted by eriophyid mites (Aceria tulipae). Double infections of MDMV and WSMV in maize have been recorded by Ford and Lambe (1967); Moline (1973) and Hill et al. (1974). Gates (1970) in Canada has observed that mites could transfer WSMV from maize to wheat and both crops act as sources of virus for the next season. He also observed that WSMV was recovered from wheat and maize plants that did not show any symptoms. Latent infections of WSMV have also been recorded by Hill et al. (1974). How (1963) has reported that WSMV was not seed transmitted in maize. Later Hill et al.(1974) reported 0,1% seed transmission of WSMV in maize.

Numerous other viruses affecting maize have been reviewed by Gordon et al. (1981) and will not be reviewed here.

TABLE 4 : OTHER FILAMENTOUS VIRUSES INFECTING MAIZE

A. Wheat streak mosaic virus.<sup>1</sup> (WSMV)

Morphology  
 Filamentous particle, 700nm by 15nm

Hosts  
 Wheat, Bromus, maize  
 Does not systemically infect sugarcane, sorghum

Transmission  
 Mechanically through sap

Sedimentation coefficient  
 165S

Genome  
 ssRNA

MW  
 2,8 x 10<sup>6</sup> d

Protein  
 45Kd (Lane and Skopp, 1983)

MW  
 Virus induces pinwheel inclusions

Situation in cells

B. Barley yellow mosaic virus.<sup>2</sup> (BYMV)

Morphology  
 Filamentous virus  
 Two modal lengths, 275nm and 550nm by 13nm

Hosts  
 Barley  
 Does not infect wheat and oats

Transmission  
 Mechanically through sap  
 Soil-borne  
 Vector probably Polymyxa graminis

Situation in cells  
 Induces crystalline bodies and pinwheel type inclusions

C. Guinea grass mosaic virus.<sup>3</sup> (GGMV)

Morphology

Flexuous filamentous virus  
825nm x 15nm

Hosts

Gramineae, including maize, sorghum

Transmission

Sap

GGMV-B and GGMV-D transmitted non-persistently  
by aphids (R.maidis)

Genome

6% nucleic acid

Protein

GGMV-A 32,5Kd  
GGMV-B 34,5Kd  
GGMV-D 32,5Kd

- 
1. Information from Brakke (1971)
  2. Information from Inonye and Saito (1975)
  3. Information from Lamy et al. (1979), Kukla et al. (1982)

CHAPTER III  
MATERIALS AND METHODS

A. BUFFERS AND REAGENTS

Buffers were made in 2x glass distilled water (gdw) to the desired molarity and pH. Buffers were stored at 22°C or at 4°C. Chemicals used were ( ) extra pure fine, GR or Analar reagent grade and were ( ) supplied by Merck or BDH.

1. GENERAL BUFFERS

Buffers for inoculation and resuspension of purified virus were potassium phosphate ( $KPO_4$ ) or borate buffers.

1(a) Potassium phosphate buffer

This buffer was made according to Williams and Chase (1968) (buffer no. 27).

Stock solutions

Solution A: 0,5M  $KH_2PO_4$  (anhydrous)

Solution B: 0,5M  $K_2HPO_4$  (anhydrous)

1(a)(i) 0,1M Potassium phosphate pH 7,0

To 78ml solution A, add 122ml of solution B and dilute to a final volume of 1 litre with gdw.

(ii) 0,1M Potassium phosphate pH 7,5

To 32ml solution A, add 168ml of solution B and

dilute to a final volume of 1 litre with gdw.

1(b) Borate buffer

This buffer was made according to Williams and Chase (1968) (buffer no. 15).

Stock solutions

Solution A: 0,2M  $H_3BO_3$  + 0,2M KCl

Solution B: 0,2M NaOH

0,5M Borate buffer pH 8,1

To 250ml solution A, add 24,5ml of solution B and dilute to 1 litre with gdw.

1(c) Extraction buffer

0,1M  $KPO_4$ /0,01M EDTA/1%  $Na_2SO_3$  pH 7,2

0,1M  $KPO_4$  pH 7,0 (III.A.1(a)(i)) containing 0,01M ethylene diamine tetra acetic acid, di-sodium salt (EDTA) and 1% (w/v)  $Na_2SO_3$ . The final pH of the buffer was pH 7,2.

1(d) Sodium phosphate buffer

This buffer was made according to Williams and Chase (1968) (buffer no. 33A). The buffers detailed below were used for isolation of Fraction I from maize plants (0,1M  $NaPO_4$  pH 7,2) and for isolation of IgG from rabbit antisera.

Stock solutionSolution A: 0,2M  $\text{NaH}_2\text{PO}_4$  (anhydrous)Solution B: 0,2M  $\text{Na}_2\text{HPO}_4$  (anhydrous)(i) 0,1M Sodium phosphate pH 7,2

To 140ml solution A, add 360ml of solution B and dilute to 1 litre with gdw.

(ii) 0,1M Sodium phosphate pH 7,4 (0,1M  $\text{NaPO}_4$  pH 7,4)

To 95ml solution A, add 405ml of solution B and dilute to 1 litre with gdw.

1(e) Phosphate buffered saline (PBS) pH 7,4

Mix equal volumes of 0,1M  $\text{NaPO}_4$  pH 7,4 (III.A.1(d)(ii)) and 0,15M  $\text{NaCl}$ .

2. BUFFER FOR ZONE ELECTROPHORESIS0,1M Borate buffer pH 8,6 (Van Regenmortel 1972)

This buffer consisted of 0,035M  $\text{H}_3\text{BO}_3$ , 0,0175M  $\text{NaOH}$ , 0,0075M  $\text{HCl}$ , 0,0037M  $\text{NaCl}$ .

Solution A: 0,2M  $\text{H}_3\text{BO}_3$  and 0,068M  $\text{NaOH}$ Solution B: 0,1N  $\text{HCl}$ Solution C: 0,15M  $\text{NaCl}$  (normal saline)

Mix 350ml solution A, 150ml solution B, 500ml solution C and 1 litre of gdw.

### 3. OUCHTERLONY DOUBLE DIFFUSION TEST BUFFERS

3(a) 0,7% agar (Bacto-Noble agar, Difco, U.S.A.) was made in phosphate buffered saline containing equal volumes of 0,1M  $KPO_4$  pH 7,0 and 0,15M NaCl. 0,1% (w/v) sodium azide was added as a preservative.

3(b) Agar gels containing sodium dodecyl sulphate (SDS) 0,8% (w/v) agar gels were made in solution containing 0,5% (w/v) SDS and 1% (w/v) sodium azide.

### 4. BUFFERS FOR ELECTRON MICROSCOPY

#### 4(a) Negative stains

(i) 1% (w/v) Ammonium molybdate pH 7,0

(ii) 2% (w/v) Phosphotungstic acid pH 7,0

(iii) 2% (w/v) Uranyl acetate pH 5,0

#### 4(b) Buffers for immunosorbent electron microscopy (ISEM)

0,076M phosphate pH 6,5 (Roberts 1980)

This buffer was used to dilute antiserum and to wash grids. The quantities used to make this buffer are specified by Williams and Chase (1968) (buffer no. 28, Sørensen's buffer).

Solution A: 0,067M  $\text{KH}_2\text{PO}_4$

Solution B: 0,067M  $\text{Na}_2\text{HPO}_4$

Mix 732ml of solution A and 268ml of solution B.

5. BUFFERS FOR ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

5(a) Coating buffer

Phosphate buffered saline (PBS) pH 7,0

Mix equal volumes of 0,1M  $\text{KPO}_4$  pH 7,0 (III.A.1(a)(i)) and saline.

5(b) Wash buffer, PBS-Tween-20

PBS containing 0,05% (v/v) Tween-20 (polyoxysorbital monolaurate, Merck Cat. No. 822184).

5(c) PBS-Tween-BSA

0,2% (w/v) bovine serum albumin (Miles Laboratories) suspended in PBS-Tween-20, containing 0,1% (w/v)  $\text{NaN}_3$ . Store at 4°C.

5(d) Conjugate

Alkaline phosphatase coupled gamma globulin was prepared as described by Clark and Adams (1977) (See also section III.D.3(c)).

5(e) Substrate solution

1mg/ml p-nitrophenylphosphate (Merck Cat. No. 6850) in

10% diethanolamine was made just before use. The solid was stored at 4°C.

5(e) (i) Substrate buffer, 10% diethanolamine

Dilute 100ml diethanolamine (Merck Cat. No. 803116) to 600ml with gdw. Adjust to pH 9,8 with concentrated HCl and dilute to a final volume of 1 litre. Store in the dark at 22°C.

6. BUFFERS FOR ELECTROPHORESIS

6(a) Polyacrylamide gel electrophoresis (PAGE) buffers

(i) Acrylamide monomer stock solution (30% (w/v) acrylamide: 0,8% (w/v) bis-acrylamide).

Dissolve 150g acrylamide (Merck Cat. No.80030) and 4,0g bis-acrylamide (Merck Cat. No. 805968) in a final volume of 500ml gdw. Store at 4°C.

(ii) Resolving gel buffer (1M Tris-HCl pH 8,8)

Dissolve 60,6g Trishydroxymethylaminomethane (tris) in 300 ml gdw. Adjust with 11,6N HCl to pH 8,8 and dilute to 500ml with gdw. Store at 4°C.

(iii) Stacking gel buffer (1M Tris-HCl pH 6,8)

Dissolve 60,6g Tris in gdw and adjust to pH 6,8 with 11,6N HCl. Dilute to 500ml with gdw and store at 4°C.

- (iv) 10% (w/v) SDS (sodium dodecyl sulphate)
- (v) 80% (v/v) glycerol
- (vi) 1,5% (w/v) Ammonium peroxodisulphate (Initiator)  
Dissolve 0,15g ammonium peroxodisulphate in 10ml  
gdw, just before use.
- (vii) Temed  
N, N, N', N' - tetramethylethylenediamine, Merck  
reagent 10732, used undiluted.
- (viii) Bath Buffer  
0,05M Tris, 0,188M glycine and 1% SDS pH 8,3.
- 10x Stock solution
- Dissolve 30,3g Tris, 141,0g glycine and 10g SDS  
in a final volume of 1 litre gdw. Store at room  
temperature. Dilute 1/10 with gdw just before use.
- (ix) Dissociation buffer  
10% SDS, 10%  $\beta$ -mercaptoethanol, 15% glycerol,  
0,01 bromophenol blue (BPB) in 1M Tris pH 6,8  
(III,A,6(a)(iii)).

Mix: 5g SDS

5ml  $\beta$ -mercaptoethanol

7,5ml glycerol

6,3ml IM Tris pH 6,8

2,5ml of a 0,2% (w/v) BPB solution

and dilute to a final volume of 50ml with gdw.

(x) Coomassie brilliant blue stain

45% methanol, 10% glacial acetic acid, 45% water  
(all v/v), and 0,2% (w/v) Coomassie blue R250  
(BDH).

Dissolve 2g Coomassie blue in 450ml methanol by  
heating gently. Filter excess dye and add 100ml  
glacial acetic acid. Dilute to a final volume  
of 1 litre with gdw.

(xi) Destaining solution

25% methanol, 10% glacial acetic acid and 65%  
gdw (all v/v).

6(b) Immunoelectroblotting (IEB) Buffers

- (i) Proteins were electroblotted onto nitrocellulose  
paper (0,45  $\mu$ m pore, Schleicher and Schuell  
BA 85, U.S.A.).

(ii) Electrophoresis transfer buffer

25mM Tris, 192mM glycine and 20%(v/v) methanol  
pH 8,3.

10x Transfer buffer stock solution

2,5mM Tris and 19,2mM glycine

Dissolve 30,28g Tris and 144,1g glycine in a final  
volume of 1 litre gdw.

(iii) 1M Tris-HCl pH 7,4

This buffer was used as a stock solution for Tris  
buffers given below. Dissolve 121,1g Tris in water.  
Adjust to pH 7,4 with 1N HCl and dilute to a final  
volume of 1 litre with gdw.

(iv) Saline (0,15M NaCl)

a 10x stock solution was prepared by dissolving  
87g NaCl in gdw to a final volume of 1 litre.

(v) Tris-saline-BSA pH 7,4

2% (w/v) bovine serum albumin (BSA, Miles Labor-  
atories) was suspended in a solution composed of  
0,01M Tris-HCl pH 7,4 and 0,15M NaCl.

(vi) Wash solution

Saline or saline containing 0,05% (v/v) Tween-20.

(vii) Labelled antiserum conjugate

Goat anti-rabbit horseradish peroxidase conjugate  
(GAR-HRP, Miles Laboratories).

(viii) Substrate 1

25ug/ml 3,3' dimethoxybenzidine (O-dianisidine)  
(Sigma), 0,01% (w/v)  $H_2O_2$ , 0,01M Tris-HCl pH 7,4.

Stock solutions

(1) 10mg/ml O-dianisidine in pure methanol.

Store in the dark at 22°C.

(2) 30% (w/v)  $H_2O_2$  (Merck)

To make substrate

Use 1/400 dilution of (1), 1/3000 of (2) and 1/100  
of 1M Tris-HCl pH 7,4 (III.A.6(b)(iii)). Make up  
volume with gdw.

(ix) Substrate 2

0,5mg/ml 4-chloro-1-naphthol (BioRad Laboratories),  
0,015% (v/v)  $H_2O_2$ , 42mM Tris-HCl pH 7,4, 167mM NaCl.

Stock solutions

(1) 3mg/ml 4-chloro-1-naphthol made up in methanol  
(Merck, Analar grade).

Store powdered chemical at -20°C and stock  
solution at 4°C.

(2) 30% (w/v) H<sub>2</sub>O<sub>2</sub> (Merck)

(3) 0,05M Tris-HCl pH 7,4 and 0,2M NaCl

To make substrate

Add 1 part (1) to 5 parts (3) and add (2) to a final dilution of 1/2000.

B. MAINTENANCE, PROPAGATION AND SOURCE OF VIRUS ISOLATES

1. VIRUS SOURCE

1(a) Virus isolates from maize

Maize dwarf mosaic virus (MDMV) isolates used in this study were obtained from field grown maize (Zea mays L.) exhibiting mosaic symptoms and were collected by Professor M B von Wechmar. The isolates are referred to as the Krugersdorp isolate or MDMV-Kru obtained from Krugersdorp in the Transvaal, the Barrow isolate or MDMV-Bar obtained from Dr Barrow, Greytown in Natal and the Winburg isolate or MDMV-Win obtained from Winburg in the Orange Free State.

A sugarcane mosaic virus strain i.e. SCMV-4975 was kindly provided by Dr A G Gillaspie Jr. from Beltsville Maryland, U.S.A. The culture was started from desiccated, infected Rio sorghum (Sorghum bicolor (L)

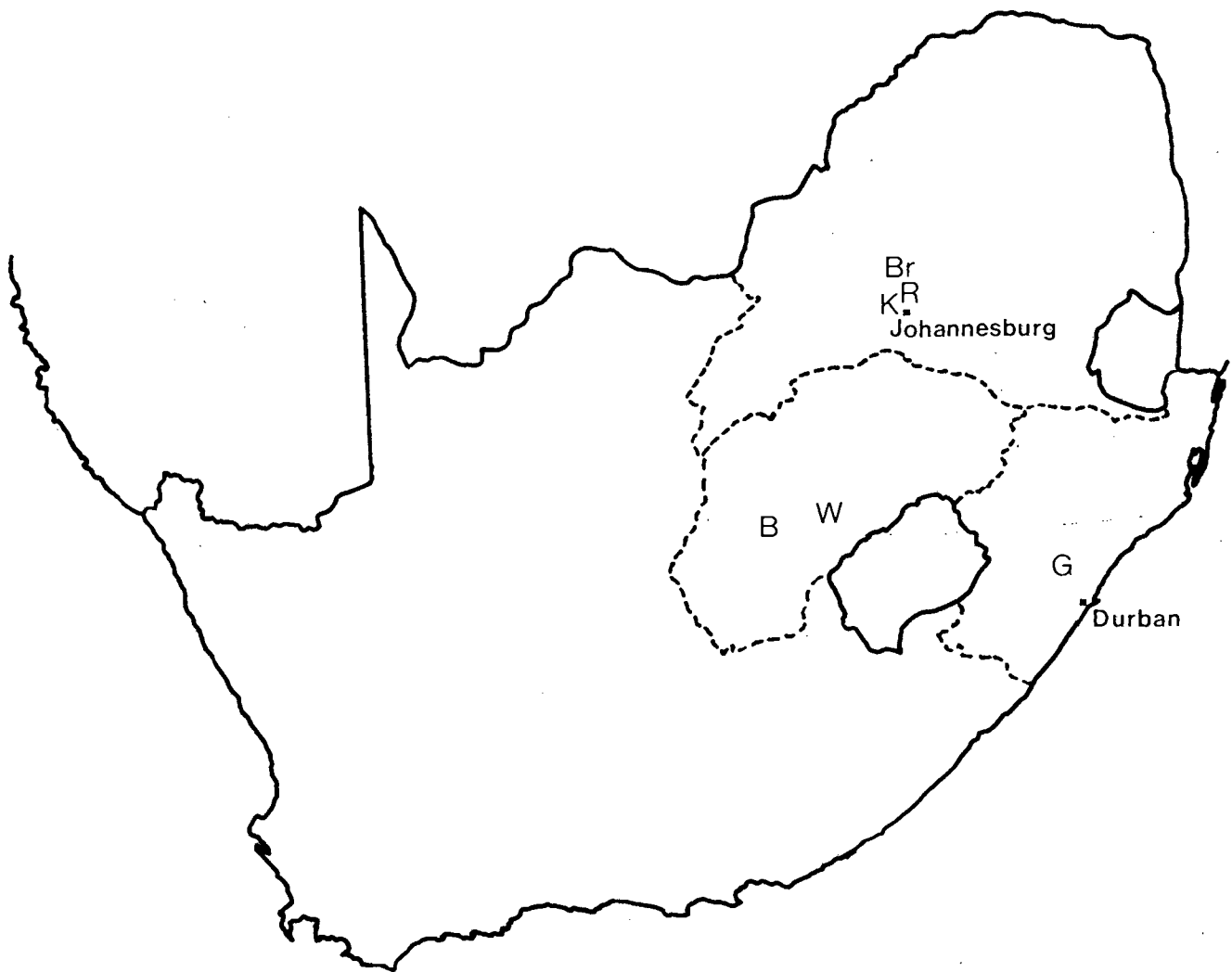


Figure 1 :

Map of South Africa showing the localities where field collected isolates were obtained:

| <u>Symbol</u> | <u>Place name</u> | <u>Isolate name</u>    |
|---------------|-------------------|------------------------|
| B             | Bloemfontein      | Abrahamskraal          |
| Br            | Brits             | Johnsongrass-isolate-1 |
| G             | Greytown          | MDMV-Bar               |
| K             | Krugersdorp       | MDMV-Kru               |
| R             | Rhodeplaat        | Rhodeplaat             |
| W             | Winburg           | MDMV-Win               |

Moench) leaves. A fifth isolate, referred to as the seed-transmitted isolate or MDMV-ST, was obtained from maize cv. Kalahari Early Pearl-Witplat. (See chapter on Seed transmission).

1(b) Virus isolates from small grains

An isolate was obtained from a naturally infected bromus grass (Bromus catharticus) plant growing alongside the road near Abrahamskraal west of Bloemfontein. The leaves exhibited mosaic symptoms as well as typical phytotoxic symptoms caused by the aphid Diuraphis noxia (Von Wechmar and Rybicki 1981). This isolate is referred to as 'Abrahamskraal' and was propagated on wheat cv. Scheepers.

\*

2. STORAGE OF VIRUS ISOLATES

Aliquots of all the MDMV isolates were stored as dessicated maize leaves (maize cv. Kalahari Early Pearl-Witplat), under anhydrous silica gel.

2(a) Dessication of samples

Infected maize leaves, harvested 2 weeks post-inoculation were dried between layers of self-indicating silica gel in a glass vacuum dessicator. The samples were left under vacuum for several days with a change of silica gel after the 1st day. The dried leaves

\* For Johnsongrass isolates -1 and -2, see sections IVB2 a and b. For Barley isolates -1 and -2, see sections IVB3 a and b.

were finally stored under silica gel in sealed standard containers at 4°C.

2(b) Storage under liquid nitrogen

For long term preservation maize isolates were stored in a cryostat (Union Carbide Liquid Nitrogen refrigerator). Freshly cut leaf pieces showing mosaic symptoms were packed in 1ml Eppendorf vials.

3. SEED SOURCE

Maize seed (cv. Kalahari Early Pearl-Witplat) is a discontinued commercial seed cultivar and was obtained from the Agricol Seed Company. The same seed source was used throughout the project. The seed was stored at 4°C and no deterioration was noticed in its germination over 2 years. Wheat cv. Scheepers and barley cv. Clipper were commercial cultivars obtained from 'Sentraal-Wes-Koöperatief', Orange Free State and Agricol Seed Company from the Western Cape respectively.

4. MAINTENANCE AND PROPAGATION OF VIRUS

All MDMV isolates were propagated in maize cv. Kalahari Early Pearl-Witplat. The barley isolates were maintained and propagated in barley cv. Clipper. The Abrahamskraal isolate was propagated in wheat cv. Scheepers. The

original Bromus plant was maintained as a "stock culture".

4(a) Plant room conditions

Plants were grown under Grolux WS VHO tubes at 1290 Watt at 16 hr light, 8 hr dark cycles in plant growth rooms. The temperature range was controlled at a maximum of 25°C and a minimum of 21°C and the relative humidity was kept at  $\pm$  70%. Plants were routinely watered twice a day and sprayed routinely with insecticide once a week. Insecticides used were Dazzle Efekto or Metasystox-R Bayer based on active ingredients, diazinon and oxydemeton-methyl respectively.

4(b) Pregermination of maize seed

Maize seeds were pregerminated for 2 days in moist vermiculite at 30°C in a Labotec incubator. The seedlings were planted four to a pot (11cm diameter) in steam-sterilized soil. Maize seedlings were separated into faster and slower germinating seeds before planting out. Only the faster germinating seedlings, later referred to as 'fast germinating maize', were used in order to obtain uniform sized maize plants for virus propagation.

4(c) (i) Production of virus-free maize plants

After finding that the maize propagation host (cv.

Kalahari Early Pearl-Witplat) contained a small percentage of seeds infected with seed-transmitted virus, it was necessary to obtain virus-free plants which could be used as controls for enzyme linked immuno-assays (ELISA), polyacrylamide gel electrophoresis (PAGE) and immuno-electroblotting (IEB) assays. Virus-free maize plants were grown by S. Leivers working on a tissue-culture project in the department. Maize plants were grown from apical meristem tip culture (personal communication, Professor M. B. von Wechmar).

(ii) Virus-free sugarcane plants

The tissue culture method was used to obtain virus-free sugarcane plantlets. These were then further cloned and planted out. These virus-free sugarcane plants were produced in the department but this was part of another project.

4(d) Inoculum preparation

Fresh infected maize leaves were ground in a heat sterilized (overnight at 110°C) mortar and pestle in the presence of heat sterilized carborundum and inoculation buffer, which was either 0,05M potassium phosphate pH 7,0 or 0,05M borate pH 8,1 (III.A.1(B)). Alternatively fresh leaves were crushed in the presence of buffer in a Pollähne press.

Dessicated leaf samples were first crushed to a powder in a mortar and pestle before addition of buffer and was left to hydrate for 15 minutes. The plant sap was filtered through cheese-cloth and celite was added to serve as abrasive. The inoculum was rubbed onto leaves using cottonwool swabs. Maize seedlings were inoculated at the 2 - 3 leaf stage, i.e. 3 - 7 day old seedlings. Plants were rinsed with tap water to remove excess celite and inoculum.

#### 4(e) Harvesting

Maize plants were harvested 14 days after inoculation and were stored short-term at 4°C prior to extraction.

### C. PURIFICATION

#### 1. CENTRIFUGATION

The term 'low speed' (L.S.) refers to centrifugation at 7000 rpm for 10 - 15 minutes in a GSA or a GS-3 rotor (at 7996g and 8288g respectively) in a Sorvall RC-5 Superspeed refrigerated centrifuge. For small volumes the SS-34 rotor was used for centrifugation at 10 000 rpm for 10 minutes at 12 062g. 'High speed' (H.S.) refers to centrifugation at 30 000 rpm for 90 minutes in a Type

30 or 35 rotor (105 700g and 105 000g respectively) in a Beckman L3-50 or L5-65 ultracentrifuge.

## 2. PURIFICATION PROCEDURE

The purification procedure that was used for extraction of MDMV is based on clarification in chloroform and concentrating the virus by polyethyleneglycol (PEG) MW 6000 precipitation.

The procedure was adapted from the methods used for purification of two other Potyviruses, Guinea-grass mosaic and papaya ringspot viruses by Lamy et al (1979) and Gonsalves and Ishii (1980), respectively.

Systemically infected maize leaves were cut into 2,5cm pieces and were homogenized in extraction buffer consisting of 0,1M  $KPO_4$  pH 7,0/0,01M EDTA/1%  $Na_2SO_3$  (III.A.1(c)) at 1 : 1 w/v ratio in a Waring blender. The homogenate was filtered through cheesecloth and the pH was observed to be at pH 7,02. After LS centrifugation the supernatant fluid was emulsified in chloroform (20ml : 100ml homogenate) for 15 to 30 minutes. The emulsion was centrifuged at LS and the pale green/yellow super-

natant was concentrated by adding 10% w/v powdered PEG 6000 and 3% w/v NaCl. The mixture was stirred for 1 hour or until the PEG had dissolved. The virus was precipitated by LS centrifugation and the precipitates were resuspended in 0,1M  $KPO_4$  pH 7,0 (III.A.1(a)(i)) to one-tenth of the original volume. The suspension was centrifuged at LS to remove aggregated, denatured host precipitates, followed by HS centrifugation. The virus pellets were resuspended in 0,05M borate buffer pH 8,1 (III.A.1(b)) or 0,1M  $KPO_4$  pH 7,0. The pellets were left in buffer at 4°C overnight. After resuspension, the virus was centrifuged at LS.

### 3. VIRUS YIELD

The concentration of virus was calculated by measuring the UV absorbance in a Beckman Model 25 spectrophotometer in a 1,2ml quartz cuvette with a light path of 1cm. The virus was diluted in either borate or phosphate buffer and read against blanks of the same buffer. The virus was scanned at wavelengths ranging from 240 - 320nm to obtain an absorbance profile. The concentration of virus was calculated using an extinction coefficient of  $E_{260}^{0,1\%} = 2,7$  (Langenberg 1973).

The yield of virus ranged from 30 - 200mg/kg of fresh

infected leaves.

#### 4. FURTHER PURIFICATION

The virus was further purified by zone electrophoresis through a sucrose gradient or by rate zonal centrifugation.

##### 4(a) Zone electrophoresis

Zone electrophoresis is a useful method for preparing virus that is free of plant material (van Regenmortel 1964) and is a method that can be used for purifying viruses without causing large losses due to aggregation as when using differential centrifugation (van Regenmortel 1966). It is a useful qualitative as well as quantitative method of preparing antigens. (Virus produced by zone electrophoresis and rate zonal centrifugation was used as immunogen in the production of antisera).

This method has been reviewed by Polson and Russell (1967) and by van Regenmortel (1972), giving exact details of buffers, apparatus and method used. The buffer system used here was 0,1M borate pH 8,6 (III.A.2) and virus applied to the sucrose gradient column was suspended in borate pH 8,1. After electrophoresis at 20mA for 18 hours, the virus fraction was collected and dialysed against half-strength borate pH 8,1.

The virus was centrifuged at HS and the pellets re-suspended in 0,05M borate pH 8,1.

4(b) Rate zonal centrifugation

To separate virus from residual host components, it was layered on a 20 - 50% sucrose density gradient made up in borate pH 8,1. The virus was centrifuged at 26 000 rpm for 2,5 hours in a Beckman SW27 or SW28 rotor. The relative centrifugal force exerted was 125 000g or 130 000g for each rotor respectively. An opalescent band was observed at approximately 2,5 - 3cm from the meniscus. The tube contents were scanned through an Isco gradient fractionator at 254nm. The fraction showing a peak was dialysed against half-strength borate pH 8,1 at 4°C overnight, concentrated by a cycle of HS centrifugation and resuspended in borate pH 8,1.

4(c) Isopycnic density gradient centrifugation

To determine the buoyant density, rate zonal purified virus was centrifuged to equilibrium in a self-generating CsCl gradient. The virus was suspended in a solution of CsCl (Merck, Suprapur) at a density of 1,36g/cm<sup>3</sup> in 0,1M KPO<sub>4</sub> pH 7,0. The CsCl-virus mixture was centrifuged for 23 hours at 40 000 rpm at 192 000g in a Beckman SW50.1 rotor. A single opalescent band was observed. The tube contents were fractionated by

puncturing the bottom of the tube and collecting either 5 or 10 drop fractions. The refractive index of each fraction was read on an Abbé refractometer (Atago) and the density was calculated using the International Critical Tables (from Beckman Instruments Publication DS-468-E). The fractions were diluted with 1ml distilled water and the absorbance was read at 260nm and 280nm on a Beckman Model 25 spectrophotometer.

#### D. ANTISERUM PRODUCTION

##### 1. METHOD OF IMMUNIZATION

Rabbits were injected intramuscularly with a 1 : 1 ratio of virus and Freund's incomplete adjuvant. Purified virus was injected into rabbits either after the differential centrifugation step or after further purification by sucrose density gradient centrifugation or zone electrophoresis. Rabbits were injected once a week for 2 to 3 weeks followed by booster injections at intervals of 3 - 6 weeks. When virus was available, further booster injections were given at 6 - 8 week intervals. One rabbit was injected with Fraction I protein, which was isolated from maize as described in section III.D.3(a) (ii). The suspension used as immunogen had an UV absorbance reading at 280nm of 0,92. Four injections at intervals of 2 to 4 weeks were administered.

## 2. COLLECTION OF SERUM

The rabbits were bled weekly from a marginal ear vein after the first two or three injections. About 20 - 30ml of blood was collected at each bleeding. The blood was allowed to clot at room temperature, followed by incubation at 4°C overnight. The serum was separated by LS centrifugation and was stored long-term at -20°C in approximately 10ml aliquots.

## 3. PREPARATION OF ANTIBODIES

### 3(a) Absorption of antisera with host proteins.

Antisera were absorbed with either maize plant sap or concentrated extract of Fraction I protein from healthy maize plants.

(i) Plant sap was prepared by homogenizing maize plants in 1 : 1 w/v 0,05M  $KPO_4$  pH 7,0 buffer in a Waring blender. The homogenate was filtered through cheesecloth and centrifuged at LS.

(ii) Extraction of Fraction I protein (Ribulose 1,5-biphosphate carboxylase complex).

All the steps were done at 4°C. Healthy maize plants were homogenized in a Waring blender 1 : 1 w/v in cold 0,05M  $NaPO_4$  pH 7,2 (III.A.1(d) (i)). The homogenate was filtered through cheesecloth

and centrifuged at LS. This was followed by HS centrifugation of the supernatant for 3 hours at 50 000 rpm at 251 800g in a Beckman 60Ti rotor. The pellets were covered in 1/20 of original volume in 0,05M NaPO<sub>4</sub> pH 7,2 and were left at 4°C overnight. The pellets were re-suspended and centrifuged at LS.

Antiserum was mixed 1 : 1 or 2 : 1 with plant sap or 3 : 1 with the extract of Fraction I protein in a conical flask. This was incubated at 37°C for 30 - 45 minutes in a water bath, with occasional stirring. The antibody-host complex was centrifuged down at LS. The absorption step was repeated once by adding plant sap or Fraction I protein at 1 : 5 ratio to the antiserum. The absorbed serum was tested in the Ouchterlony gel diffusion test against healthy plant sap. Antiserum was re-absorbed with plant sap if the antisera showed a reaction with plant sap.

3(b) Production of gamma-globulin by ammonium sulphate precipitation

Antisera of a titre of 1/512 or 1/1024, as determined by the tube precipitation test were used to produce purified gamma-globulin. Saturated

ammonium sulphate was mixed at 1 : 1 ratio to precipitate the absorbed serum. The ammonium sulphate was added dropwise with constant stirring. The mixture was allowed to stand for 15 minutes at 22°C. After LS centrifugation the precipitate was resuspended in 1/5 of the original volume with saline. This was diluted with water to the original volume and re-precipitated with an equal volume of saturated ammonium sulphate. After LS centrifugation the precipitate was resuspended in 1/4 the original volume with saline containing 0,1% sodium azide. The gamma-globulin was dialysed against 2 litre volumes of saline containing 0,1% sodium azide at 4°C overnight with one change of saline. The partially purified gamma-globulin was stored at -20°C in 5 to 10ml aliquots.

The gamma-globulin was further purified by ion-exchange chromatography by filtering through a diethylaminoethyl-cellulose column (Whatman DE52). The DEAE-cellulose was equilibrated in half-strength PBS pH 7,4 (III.A.1(e)) and packed into a 15 x 1cm glass column. The gamma-globulin was layered on the column and washed through with half-strength PBS pH 7,4. 1ml fractions were collected and the U V absorbance at 280nm was read on a spectrophotometer. The fractions giving the highest absorb-

ance readings were pooled and the concentration of the purified gamma-globulin was adjusted to  $1\text{mg/ml}$  with half-strength PBS pH 7,4. The extinction coefficient  $E_{280}^{0,1\%}$  of gamma-globulin was taken to be 1,4 (Clark and Adams 1977).

### 3(c) Conjugation with alkaline phosphatase

The alkaline phosphatase was conjugated to gamma-globulin in the presence of glutaraldehyde (Merck 4239, 25%) in a one-step procedure (Clark and Adams 1977). 2mg alkaline phosphatase, a chromatographically purified, freeze dried powder with an activity of 1 000 units/mg protein (Miles) was dissolved in  $1\text{ml}$  of a  $1\text{mg/ml}$  solution of purified gamma-globulin. This was dialysed against 2 litres of PBS pH 7,0 at  $4^{\circ}\text{C}$  overnight, with one change of buffer. Glutaraldehyde was added to the alkaline phosphatase mixture to 0,05% final concentration in a sealed glass bottle. The mixture was incubated at  $22^{\circ}\text{C}$  for 4 hours. Glutaraldehyde was removed by dialysis against several changes of PBS pH 7,4 at  $4^{\circ}\text{C}$ . BSA was added to 1% final concentration to stabilise the conjugate, which was stored in a sealed glass bottle at  $4^{\circ}\text{C}$ .

## E. SEROLOGICAL METHODS

The tube precipitin and microprecipitin tests were used to

determine the antiserum titre. The microprecipitin test and the Ouchterlony double diffusion test in agar were used for the preliminary serological detection of virus.

#### 1. THE TUBE PRECIPITIN TEST

This test was done in 10 x 75mm test tubes. The antigen was plant sap clarified with 10% chloroform followed by incubation at 37°C in a waterbath for 15 - 30 minutes followed by LS centrifugation. This step allowed plant proteins to coagulate. The preparation was diluted to 1/5 or 1/10 before use. The antiserum was serially diluted two-fold in saline containing 0,1% sodium azide as a preservative. 0,25ml or 0,5ml of the two-fold dilutions of antiserum was added to a row of tubes. An equal volume of antigen was added to each dilution of antiserum. The contents were thoroughly mixed and incubated at 37°C for 2 hours, followed by incubation overnight at 22°C. The presence of a flocculent precipitate was observed by viewing the tubes over a lamp in a dark room. The tubes were gently rotated to disturb the precipitate sufficiently for better observation.

The highest dilution of antiserum that showed a visible precipitation was taken as the antibody titre. Controls were set up with clarified healthy plant sap.

#### 2. THE MICROPRECIPITIN TEST

This technique was preferred over the tube precipitin test

as much smaller volumes of reagents were required. This test was done in disposable 10cm diameter plastic Petri dishes. A grid of 36 blocks was drawn on the bottom of the Petri dish. Two-fold serial dilutions of purified MDMV, with a starting concentration of 2mg/ml, were made in 0,05M borate pH 8,1. Two-fold dilutions of antiserum were made in saline containing 0,1% sodium azide. 10ul drops of antiserum dilutions were placed in rows in the grid, using a 10ul fixed volume Eppendorf micropipette. The appropriate dilutions of antigen were added in columns to the antisera drops.

Antigen and antiserum controls consisted of dilutions of unmixed antigen and antiserum. The Petri dishes were covered, gently agitated and placed in moist freezer containers, closed, and incubated at room temperature followed by incubation at 4°C overnight. Reactions were observed after 15 - 60 minutes at a magnification of 25x through a Zeiss stereo microscope fitted with indirect lighting.

### 3. THE OUCHTERLONY DOUBLE DIFFUSION TEST

This test was done in 0,7% agar (Bacto-Noble agar, Difco) in disposable plastic Petri dishes. Agar was prepared in PBS, i.e. equal volumes of 0,1M  $KPO_4$  pH 7,0 and 0,15M NaCl. 0,1% sodium azide was added as a preservative. 20ml of agar was poured into each Petri dish.

The method of Purcifull and Batchelor (1977) was also tried.

SDS is incorporated into the agar to disrupt antigen, in order to facilitate diffusion of large molecules, e.g. filamentous viruses. 0,8% agar containing 1% sodium azide and 0,5% SDS was made. 4 ring patterns with 6 peripheral wells and a centre well (each 4mm in diameter), were cut into the agar with a gel cutter. The wells were formed by removing the agar pieces by suction. Dilutions of antigen were added to the peripheral wells and antiserum to the centre wells. The agar plates were incubated at room temperature in moist plastic freezer containers overnight. The formation of the precipitin bands were observed over a lightbox. Precipitin bands in the 0,7% agar were observed after 1 or 2 days incubation. Precipitin bands in the agar containing SDS were observed after 1 day incubation.

#### 4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a sensitive technique for detection of low concentrations of antigen in either purified or crude virus extracts. Two procedures, the indirect and double antibody sandwich procedures were used.

##### 4(a) Equipment

The tests were carried out in sterile flat-bottomed microtitre trays (Cooke, Dynatech or Linbro, Nunc). For all pipetting operations, the following micro-pipettes were used:

Finn variable volume micropipettes  
(0-50ul, 50-250ul, 200-1000ul)

Gilson Pipetman, variable volume  
(0-20ul)

Gilson Repetman, repeating variable  
volume micropipette (0-1000ul)

For the incubation steps, trays were incubated in moist closed plastic freezer containers at 37°C in an incubator or at 4°C.

The absorbance at 405nm, of the coloured hydrolysis product formed by the action of the enzyme on the substrate, was read on a Titertek Multiscan automatic spectrophotometer. The matrix mode on the Multiscan spectrophotometer was employed to obtain absorbance values that exceeded the 0 - 2 range of the absorbance mode.

#### 4(b) Antisera

Purified IgG and conjugated IgG were prepared as described in sections III.D.3(b) and (c). Alkaline phosphatase conjugated goat anti-rabbit IgG, which was prepared in the department, was obtained from the anti-serum collection of Microbiology Department, U.C.T. IgG was diluted in PBS-Tween-BSA (III.A.5(c)). When IgG was used for coating the wells, it was diluted in PBS pH 7,0 (III.A.5(a)).

#### 4(c) Preparation of Antigen

Virus samples tested in the ELISA were either purified virus preparations or crude plant extracts.

(i) Virus was purified as described in Section III C.

After HS centrifugation the virus was resuspended in 0,05M Borate pH 8,1 or 0,1M  $KPO_4$  pH 7,0. The samples were serially diluted four-fold in PBS-Tween-BSA for use in the double antibody sandwich procedure. When tested in the indirect procedure, virus was diluted in the appropriate buffer or PBS. Positive control was purified MDMV and the negative control used was concentrated preparations of healthy maize plants or plant sap. When available, sap of plants raised from apical meristem tip cultures was used (III.B.4(c)).

(ii) Crude preparations of infected and healthy maize plants were prepared by grinding leaves in an equal volume of 0,1M  $KPO_4$  pH 7,0 in a mortar and pestle. Sap was filtered through cheesecloth and centrifuged in a bench-top centrifuge or in a Sorvall centrifuge at LS. Extracts were diluted two-fold in PBS-Tween-BSA for use in the 'sandwich' procedure or were diluted two-fold in 0,1M  $KPO_4$  pH 7,0 or PBS for use in the indirect procedure.

#### 4(d) Procedure

##### (i) Double antibody sandwich method

###### Coating of wells

200ul of the optimal dilution (1/250 - 1/400) of 1mg/ml IgG specific for MDMV was added to the wells in a microtitre tray. The 1st column was used as a substrate control, and 200ul of PBS was added. The tray was incubated at 37°C for 2 hours. Excess antibody was emptied from the wells before washing them.

###### Washing the plate

The wells were rinsed in PBS-Tween (III.A.5(b)) and then soaked for 3 minutes in PBS-Tween. The tray was emptied and tapped on paper towelling to remove residual buffer. The wells were soaked two further times in PBS-Tween for 3 minutes each. The wells were finally soaked in PBS-Tween-BSA for 5 minutes to block any free adsorption sites.

###### Addition of antigen

200ul of the diluted antigen was added to the appropriate wells. The tray was incubated at 37°C for 2 hours or at 4°C overnight. The trays were washed.

Addition of conjugate

200ul of conjugate at optimal concentration (usually 1/300 - 1/400) was added to the wells. The tray was incubated at 4°C overnight or at 37°C for 2 hours. The trays were washed.

Addition of substrate

300ul of 1mg/ml solution of p-nitrophenylphosphate hexahydrate in 10% diethanolamine (III.A.5(e)) was added to the wells. The tray was left at 22°C for the enzyme-substrate reaction to develop. The reaction was stopped by adding 50ul of 3M NaOH. The absorbance of the yellow colour formed was read at 405nm on the Titertek Multiskan spectrophotometer.

(ii) The indirect methodAddition of antigen

200ul of the appropriate dilution of virus or control antigen was added to the wells. After incubating at 37°C for 2 hours, the trays were washed.

Addition of specific antibody

200ul of antibody specific for MDMV was added to the wells. After incubating at 37°C for 2 hours, the trays were washed.

### Addition of GAR-conjugate

200ul of 1/1000 dilution of goat-anti-rabbit conjugate was added to the wells. The tray was incubated at 4°C overnight. After washing the plates, substrate was added and the absorbance at 405nm monitored as described above.

## F. ELECTRON MICROSCOPY

### 1. NEGATIVE STAINING

Virus samples in infected leaves or purified preparations of virus were negatively stained in 2% phosphotungstic acid, 1% ammonium molybdate at pH 7,0 or in 2% uranyl acetate at pH 6,0. Carbon-coated formvar grids were placed on wax in a Petri dish and covered with a drop of virus sample. This was incubated at room temperature for 5 minutes. The grids were washed in drops of distilled water. A drop of stain was added and the grid incubated for 1 minute at 22°C. The grid was drained by touching the edge of the grid to filter paper. The samples were viewed in a Zeiss 109 transmission electron microscope at 80 KV.

### 2. IMMUNOSORBENT ELECTRON MICROSCOPY (ISEM)

This technique makes use of electron microscopy and serology, where the virus-antibody complex can be directly examined (Milne and Luisoni 1977). Derrick in 1973 introduced the technique where virus was specifically attached to antibody-

coated grids. The procedure used was that of Roberts (1980).

## 2(a) Procedure

Carbon-coated grids were floated on drops of anti-serum placed on dental wax in humid Petri dishes. The antiserum was diluted to 1/1000 in 0,06M  $KPO_4$  pH 6,5. The grids were incubated for 1 - 1,5 hours at 4°C in a moist container, washed twice in 0,067M phosphate buffer pH 6,5 (III.A.4(b)) for 10 - 15 minutes and drained with filter paper. They were then floated on drops of purified MDMV preparations or on drops of crude plant sap, incubated at 4°C for 1 - 1,5 hours in a moist container, washed in drops of water from a pasteur pipette and stained in 2% uranylacetate. After draining with filter paper, the grids were allowed to dry and viewed.

## G. ELECTROPHORESIS

### 1. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Vertical slab gel electrophoresis was performed using the discontinuous system of Laemmli (1970) in a Hoefer SE600 slab gel apparatus. The apparatus consists of glass plates 18 x 16cm arranged to hold vertical slab gels of 1,5mm thickness.

1(a) Preparation of the gel

(i) The resolving gel

A 12,5% gel was made by mixing the following:

33,3ml acrylamide stock solution (III.A.6(a)(i))

30,0ml resolving gel buffer (III.A.6(a)(ii))

11,9ml distilled water

0,8ml 10% SDS (III.A.6(a)(iv))

4,0ml 1,5% ammonium peroxodisulphate (III.A.6(a)(vi))

0,02ml Temed (III.A.6(a)(vii))

This made 80,02ml of gel solution which was sufficient to pour 2 gels, 25ml per gel. The resolving gel solution was poured between the vertical glass plates up to a height of 12cm. A layer of water was carefully applied to the top of the gel surface, to exclude oxygen which prevents the setting of the gel and to obtain a level interface. The gel was allowed to set for 30 to 60 minutes at room temperature.

(ii) The stacking gel

Gel solution was prepared by mixing the following:

4,5ml acrylamide stock solution

3,8ml stacking gel buffer (III.A.6(a)(iii))

18,0ml distilled water  
2,0ml 80% glycerol  
0,3ml 10% SDS  
1,4ml 1,5% ammonium persulphate  
0,04ml Temed

The water layer was poured off and the stacking gel solution was poured over the set resolving gel. 10 wells, each 8mm wide were cast in the stacking gel by inserting a slotted comb. The gel was allowed to set for 30 - 60 minutes at room temperature.

1(b) Dissociation of samples

Samples were either crude virus extracts or purified preparations. Healthy control extracts were prepared as the virus extracts. Crude extracts consisted of leaf material which was crushed in a mortar and pestle in an equal volume of 0,1M  $KPO_4$  pH 7,0. This was clarified by centrifuging at 5 000 rpm in a bench top centrifuge for 10 minutes or at low speed in a Sorvall centrifuge. The preparations were mixed in equal volumes of dissociation buffer (III. A.6(a)(ix)) and were heated at 95°C for 10 minutes. Dissociated samples were used immediately or stored in sealed Wasserman tubes at -20°C until needed. Marker proteins of different molecular weight

(Pharmacia) consisted of the following:

|                           |        |
|---------------------------|--------|
| Phosphorylase b           | 94 000 |
| Bovine serum albumin      | 67 000 |
| Ovalbumin                 | 43 000 |
| Carbonic anhydrase        | 30 000 |
| Soybean trypsin inhibitor | 20 100 |
| <del>α</del> Lactalbumin  | 14 400 |

1(c) Sample application and electrophoresis

After the stacking gel had set, the comb, forming the wells, was removed. The wells were rinsed with distilled water and filled with 1 x bath buffer (III.A.6(a)(viii)). 15ul to 40ul of dissociated sample was added to the wells with a Hamilton syringe. The gels were electrophoresed at 10mA per gel overnight or at 35mA per gel for approximately 5 hours at 4°C until the bromophenol blue dye front had reached the bottom of the gel.

1(d) Staining and destaining

The gels were removed from the apparatus and were placed in Coomassie blue stain (III.A.6(a)(x)) in square plastic freezer containers overnight at room temperature. The gels were placed in several changes of destaining solution (III.A.6(a)(xi)) or were placed in an apparatus containing felt absorbent and

continuously circulating buffer for 12 or more hours until most of the background stain was removed by diffusion. The gels were dried by vacuum onto Whatman 3MM filter paper on a Hoefer SE 540 gel dryer for 1,5 to 2 hours.

## 2. IMMUNO-ELECTROBLOTTING (IEB)

This is a very sensitive method for the detection of proteins, combining the electrophoretic separation of proteins with detection by an indirect enzyme-assisted immuno-assay. (Rybicki and von Wechmar 1982, and Towbin et al 1979).

The apparatus described by Rybicki and von Wechmar (1982) was used. Later on the Hoefer Transfor TE52 electrophoresis cell was also employed.

### 2(a) Electrophoretic transfer (electroblotting)

Nitrocellulose sheets (11 x 14cm) and filter paper sheets (Whatman 3MM) were wetted in transfer buffer (III.A.6(b)(ii)). The nitrocellulose sheets were layered on the filter paper. After electrophoresis the stacking gel was discarded and the resolving gel was rinsed in transfer buffer and carefully placed on the nitrocellulose sheet. Wet filter paper was layered onto the resolving gel. Care was taken in all steps to prevent trapping of air bubbles between layers. The assembly was placed between 2 Scotch-

Brite scouring pads and 2 polyethylene sheets on the outside. Two carbon electrodes (20 x 15 x 1cm) were placed on either side of the polyethylene sheets and were kept in place by elastic bands. The whole assembly was placed in 3,5 litres of transfer buffer in a tank. The electrodes were connected to a Shandon Southern 50V 1A power unit (Model No.SAE 2766) with the anode nearest the nitrocellulose. A current of 1A was applied for approximately 2 hours. Up to 5 gels could be electroblotted at a time by separating gel, nitrocellulose and filter paper assemblies with Scotch-Brite pads.

## 2(b) Enzyme immuno-assay

### (i) Incubation in antiserum

The nitrocellulose sheets (electroblots) were removed and soaked individually in Tris-saline-BSA pH 7,4 (III.A.6(b)(v)) in covered plastic freezer containers at 22°C overnight or at 40°C for 2 hours. Antiserum was diluted 1/25 - 1/100 in Tris-saline-BSA buffer. 30ml diluted antiserum was sufficient to cover an 11 x 14cm electroblot. Blots were incubated for 2 hours at 22°C on a shaker.

### (ii) Washing of the blots

The blots were washed in saline or saline contain-

ing 0,05% Tween-20 (III.A.6(b)(vi)). The incorporation of Tween-20 greatly reduced the background reaction. The blots were rinsed twice in washing solution and then washed for 30 minutes with 3 changes of saline, on a shaker.

(iii) Addition of conjugate

The labelled conjugate, goat anti-rabbit horse radish peroxidase, was diluted 1/1500 in Tris-saline-BSA. The blots were incubated for 2 hours at 22°C on a shaker (Labotec) and then washed as above.

(iv) Addition of enzyme substrate solution

Substrate containing O-dianisidine (III.A.6(b)(viii)) or 4-chloro-1-naphthol (III.A.6(b)(ix)) were used to visualize the proteins on the electroblot. 40 - 50ml was added to an 11 x 14cm blot and incubated at 22°C for 10 - 30 minutes. The blots were washed in water to stop the reaction. The dianisidine substrate solution gave a brick coloured reaction, whereas the 4-chloro-1-naphthol substrate showed a purple-blue reaction. The latter substrate is less toxic and gave a better contrast.

The blots were dried on layers of paper-towelling or filter paper and were photographed using a red filter.

## CHAPTER IV

### RESULTS

#### A. IDENTIFICATION OF MAIZE ISOLATES

##### 1. HOSTS, PROPAGATION AND SYMPTOMS

###### Description of symptoms on maize

Maize (Zea mays L.) cv. Kalahari Early Pearl (Witplat) (K.E.P.-Witplat) was used routinely (unless specified otherwise) for the propagation of the local MDMV isolates and SCMV 4975. Symptoms on maize developed 5 - 7 days post inoculation. The first symptoms that developed were pale yellow elliptical streaks starting at the base of the youngest and second leaves. This developed into a pale yellow green mosaic along the entire leaf.\* An example of the mosaic symptoms is shown in Fig. 2a and b. Generally, the 1st leaves of inoculated plants showed necrosis. Sub-inoculation from such necrotic leaves to healthy maize again produced mosaic symptoms. The necrosis could be a result of high inoculum concentration.

Other cultivars of maize were treated for susceptibility to MDMV-Kru isolate, but K.E.P.-Witplat was found to have the highest percentage (75%) of infected plants.

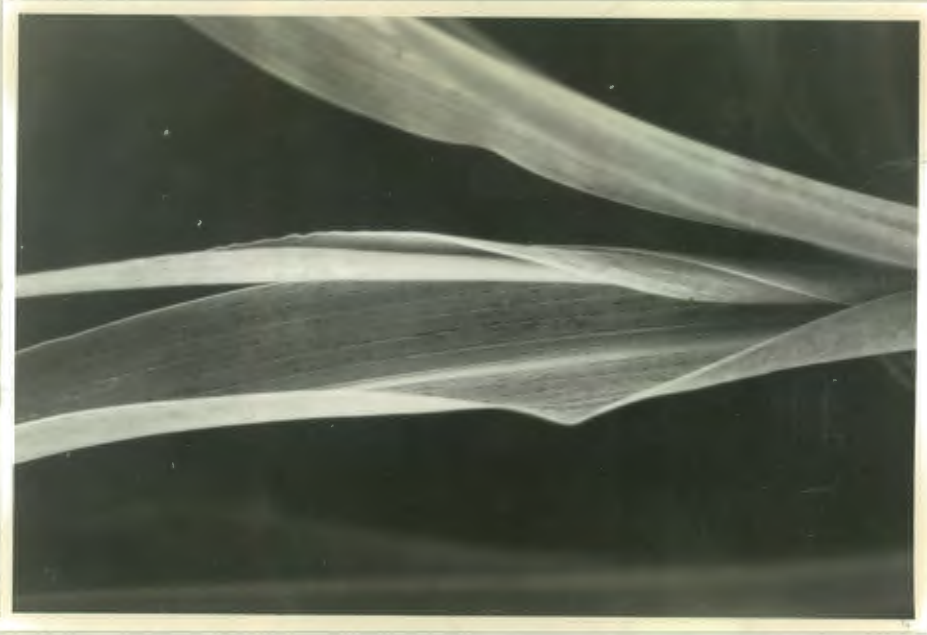
(See Table 5)

\* Different isolates could not be differentiated by symptoms only.



a

Figure 2 : a - Maize plant infected with MDMV-Kru showing mosaic symptoms (right). Plant with a dead growing point, arrow, and in the foreground, a dwarfed maize plant (second arrow)



b

b - Close up of mosaic symptoms on maize infected with MDMV-Kru

Table 5 : The percentage infection of various types  
of maize after inoculation with the MDMV-Kru  
isolate

| <u>Maize type</u>                | <u>No. of plants<br/>inoculated</u> | <u>No. of<br/>infected<br/>plants<sub>1</sub></u> | <u>% infected<br/>plants</u> |
|----------------------------------|-------------------------------------|---------------------------------------------------|------------------------------|
| Goudveld                         | 32                                  | 8                                                 | 25                           |
| Kalahari Early Pearl-<br>Witplat | 79                                  | 59                                                | 75                           |
| PNR 95                           | 43                                  | 11                                                | 25                           |
| SA 4                             | 63                                  | 21                                                | 33,3                         |
| SA 100                           | 47                                  | 23                                                | 36,5                         |
| Witmielies SR52                  | 64                                  | 32                                                | 50                           |
| <u>Sweetcorn</u>                 |                                     |                                                   |                              |
| Golden Bantam                    | 77                                  | 55                                                | 71                           |
| Stowels Evergreen                | 44                                  | 22                                                | 50                           |

1. Plants were visually inspected for the presence of mosaic symptoms.

Sorghum cv. NK 500 when inoculated with MDMV-Kru isolate produced a mosaic on 10% of the plants. A further 10% showed a hypersensitive reaction of chlorotic red spots on the leaf blade and red leaf tips.

MDMV-Kru isolate was inoculated onto barley cv. Clipper, but was not infective when inspected visually.

Initially fast and slow germinating maize seedlings were not separated (see III B.4.b). When seedlings, grown from unseparated seed batches, were inoculated, several abnormal symptoms were observed in such seedlings as listed in Table 6. The table gives data for MDMV-Kru but similar observations were made when other isolates were inoculated onto seedlings grown from unselected seed. The abnormal symptoms were mainly dwarfing and dead growing points which is shown in Fig. 2a. Occasionally single plants exhibited mosaic symptoms at a rate of 1 in 400 plants. To avoid the problem of abnormal seedlings, the practice of selecting fast germinating seed was adopted. (See Seed transmission, section IV C.).

Table 6 : The percentage of plants exhibiting symptoms other than mosaic on maize plants inoculated with MDMV-Kru.

| <u>Symptom</u>     | <u>No. of plants with abnormal symptoms</u> <sup>1</sup> | <u>Total no. of plants inoculated</u> | <u>%</u> |
|--------------------|----------------------------------------------------------|---------------------------------------|----------|
| Dwarfing           | 8                                                        | 76                                    | 10,5     |
|                    | 27                                                       | 432                                   | 6,25     |
|                    | 9                                                        | 78                                    | 11,5     |
| Dead youngest leaf | 3                                                        | 432                                   | 0,69     |
|                    | 4                                                        | 78                                    | 5,1      |

Footnote:

1. Observations made in different batches of plants.

## 2. HARVESTING

Infected maize plants, i.e. those showing mosaic symptoms were harvested 14 - 21 days post inoculation (normally 14 days post inoculation<sup>\*</sup>). Leaves were used for virus purification immediately after harvesting or were kept at 4°C for short-term storage until used.

## 3. STORAGE OF VIRUS ISOLATES

The longevity of the isolates stored in dessicated leaves (see III B.2.a) and in leaves stored in the cryostat (see III B.2.b) was tested.

### a. Infectivity of virus in dessicated leaves.

#### Rehydration of dried leaves.

A sample of dessicated leaves was ground to a fine powder in the presence of carborundum in a mortar and pestle. 5ml of 0,1M KPO<sub>4</sub> pH7,0 was added and the ground leaves allowed to stand for 15 - 30 minutes. The sap was filtered through cheesecloth and inoculated onto maize plants.

Dessicated leaves infected with MDMV-Kru were infective 1 month but not 9 months after dessication (other times not tested). MDMV-Bar, MDMV-Win and SCMV 4975 isolates were not infective after 8, 6 and 7 months' storage

\* Young plants were harvested to facilitate purification and a good yield was obtained from these plants.

respectively in the dessicated leaves.

On investigation, it was shown that moisture had got into the silica gel, so that the leaves were not completely dried. This could have resulted in the virus becoming inactive. Therefore these results are not a true indication of the longevity of the virus in dessicated leaves. (Prof. M.B. von Wechmar (personal communication) has shown that SCMV and MDMV isolates could retain infectivity for 10 years when stored as lyophilized infected host tissue).

b. Infectivity of virus stored in leaves stored under liquid nitrogen

Leaf samples removed from the cryostat were ground immediately in 0,1MKPO<sub>4</sub> pH7,0 buffer in the presence of carborundum in a mortar and pestle. The sap was left for 15 - 30 minutes and filtered through cheesecloth before inoculation onto maize plants. MDMV-seed transmitted (MDMV-ST) (see Seed transmission, section IV.C) stored for 4½ months was 100% infective. MDMV-Kru was infective after 31,2 months of storage.

#### 4. PURIFICATION

##### a. Procedure

The purification of the virus from infected maize was attempted by using four protocols from different sources. Method 4 was the most successful and was used for further routine purification. Methods 1, 2 and 3 were tried, but were not successful. These methods are given below.

Preparations after each purification step were tested for infectivity on maize seedlings.

##### Method 1

Chloroform clarification and concentration of virus by differential ultracentrifugation as adapted from von Wechmar and Hahn (1967).

MDMV-Kru infected leaves homogenized in 0,18M McIlvaine buffer pH7,24 containing 0,1% Leonil detergent, 1:3 (w/v) leaves to buffer ratio.

↓  
Clarify in 10ml chloroform per 100ml homogenate. Stir 15 minutes.

↓  
Filter through cheesecloth.

↓  
Centrifuge at low speed (L.S.) (See III C.1.)

↓  
├── discard precipitate  
↓

Supernatant centrifuged at high speed (H.S.) (See III C.1.)

↓  
Pellets resuspended in McIlvaine buffer pH7,2

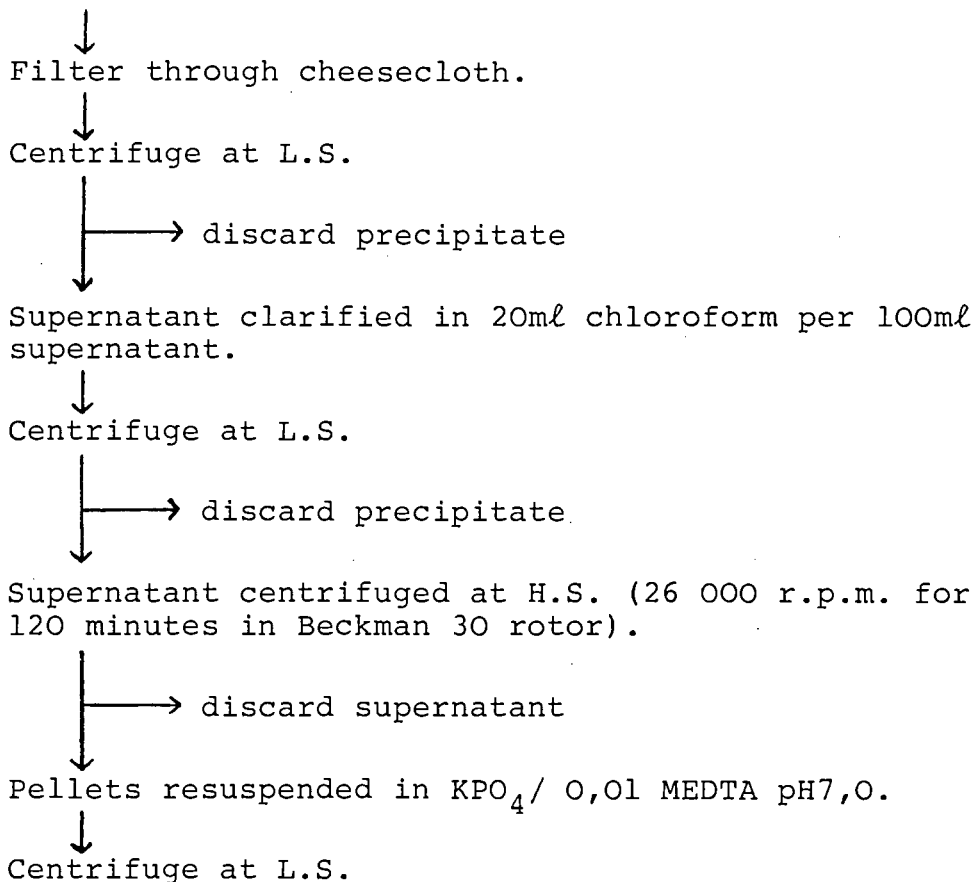
↓  
Centrifuge at L.S.

Only plants inoculated with the supernatant after the first L.S. centrifugation became infected.

Method 2

Chloroform clarification and concentration of virus by differential ultracentrifugation (Lamy et al 1979).

MDMV-Kru infected leaves homogenized in 0,1M  $KPO_4$  /0,01MEDTA/1%  $Na_2SO_3$  pH7,2 , 1:1 (w/v) leaves to buffer ratio.



The first and second L.S. preparations were infective on maize as well as the H.S. preparation. The H.S. pellets obtained were green, containing a large amount of plant proteins.

### Method 3

Chloroform, carbon tetrachloride, Triton X-100 clarification (Gough and Shukla 1981).

MDMV-Kru infected leaves were ground in 1,5 volumes of 0,5M Borate pH8,1 containing 0,15% (v/v) thioglycollic acid and 0,01M EDTA.

↓  
Filter through cheesecloth.

↓  
Clarify in  $\frac{1}{2}$  volume each of  $\text{CHCl}_3$  and  $\text{CCl}_4$ .

↓  
Centrifuge at L.S.

↓  
→ discard precipitate

↓  
Triton X-100 (alkylphenoxypolyethoxyethanol) was added to 5% (v/v) to the supernatant. Stir for 30 minutes at 4°C.

↓  
Centrifuge at L.S.

↓  
Supernatant centrifuged at H.S.

↓  
Pellet resuspended in Borate pH8,1/0,15% thioglycollic acid /0,01M EDTA.

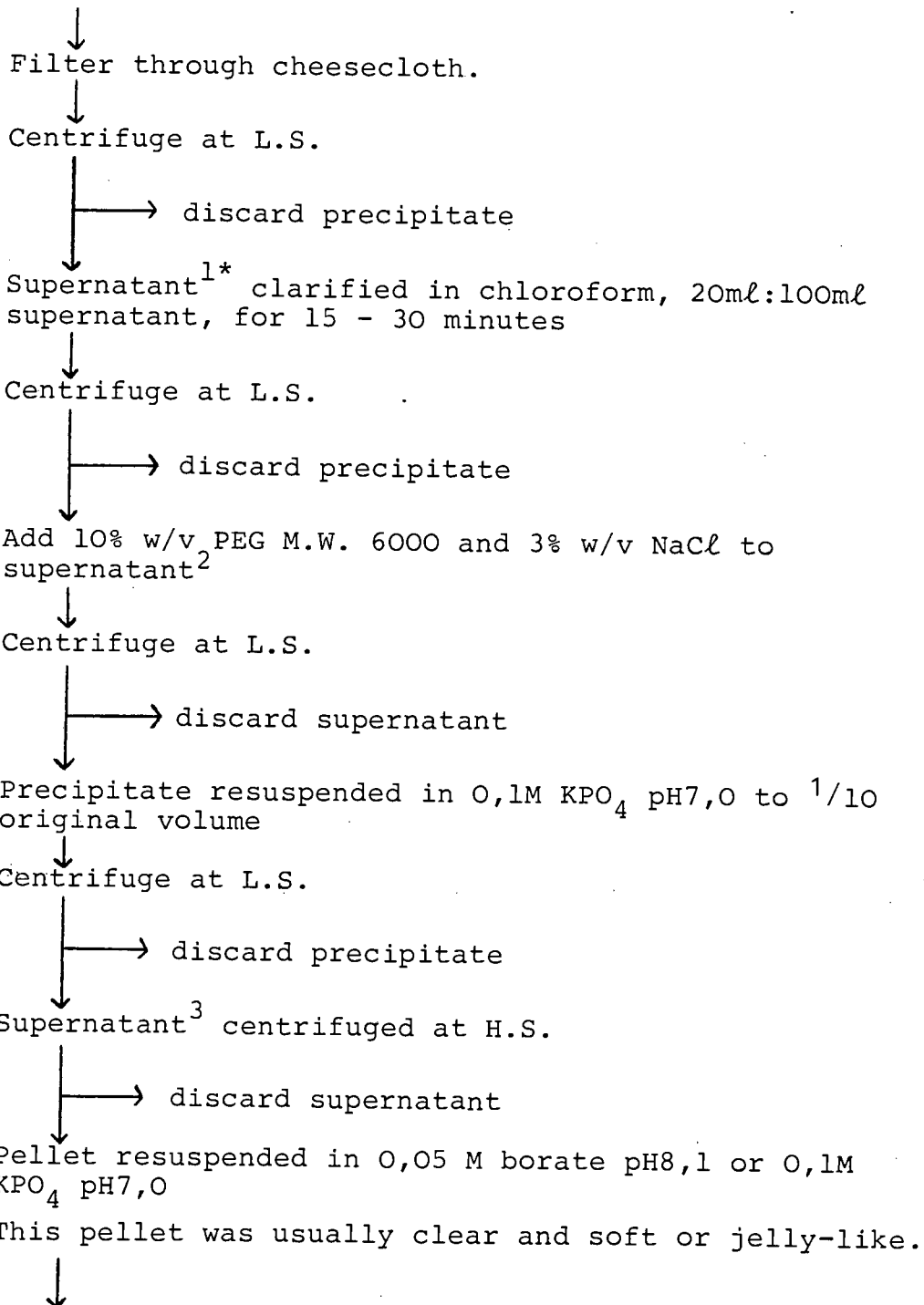
↓  
Centrifuge at L.S.

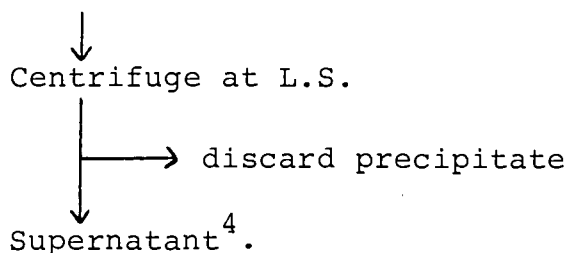
Infectivity of the preparations after Triton X-100 treatment and after H.S. centrifugation were tested by inoculation onto maize plants, but were not infective. The final preparation was subjected to rate zonal centrifugation in a sucrose gradient but no opalescent band was observed.

Method 4

Chloroform clarification, PEG precipitation and concentration by differential ultracentrifugation as adapted from Lamy et al (1979) and Gonsalves and Ishii (1980) (See III.C).

MDMV-Kru infected leaves were homogenized in 0,1M KPO<sub>4</sub> pH7,0/0,01MEDTA/1% Na<sub>2</sub>SO<sub>3</sub>, 1:1 (w/v) leaves to buffer ratio.





\* See text.

Before adopting Method 4 as above, the following variations were tried:

- (i) extraction buffer consisting of 0,1M  $KPO_4$  pH7,0  
or 0,1M  $KPO_4$  pH7,0 with additives 0,01M EDTA and 1%  
 $Na_2SO_3$  ,
- (ii) chloroform at 10ml:100ml sap or 20ml:100ml sap,
- (iii) PEG (MW 6 000) at concentrations of 9g or 10g and  
NaCl at 2,5g or 3g per 100ml clarified sap.

On comparison, the best preparations were obtained with 20ml chloroform per 100ml sap and 10g PEG MW 6 000 and 3g NaCl per 100ml clarified sap. These quantities were adopted as standard in all later purifications.

The preparations obtained at different stages of purification, as numbered in the flow diagram above, were tested for infectivity by inoculation onto maize. All the preparations were infective showing mosaic symptoms after

4 - 14 days post-inoculation with the average time for symptom development being 6 - 7 days post inoculation.

b. Virus yield

The virus yield after differential ultracentrifugation was in the range 30 - 200mg per kg fresh leaf weight, with an average of 71mg/kg  $\pm 64$  (this value was based on seven extractions). The absorbance ratio A260/A280 was in the range 1,5 - 1,77 with an average of 1,6  $\pm 0,02$ . On two occasions high virus yields of 777 and 735mg/kg fresh leaf weight, had been obtained, when isolating for virus from MDMV-Win and MDMV-Kru-infected leaves respectively. The A260/A280 ratios obtained were 1,78 and 1,77 respectively. A typical UV absorbance profile of MDMV-Kru at this level of purification is shown in Fig. 3.

c. Infectivity

The MDMV-Kru isolate was infective 1 week after isolation when stored in 0,05M borate pH8,1. 1/6 plants showed mosaic symptoms 1 week post inoculation and 2/6 plants showed mosaic symptoms 3 weeks post inoculation in contrast to 100% infectivity immediately after isolation.

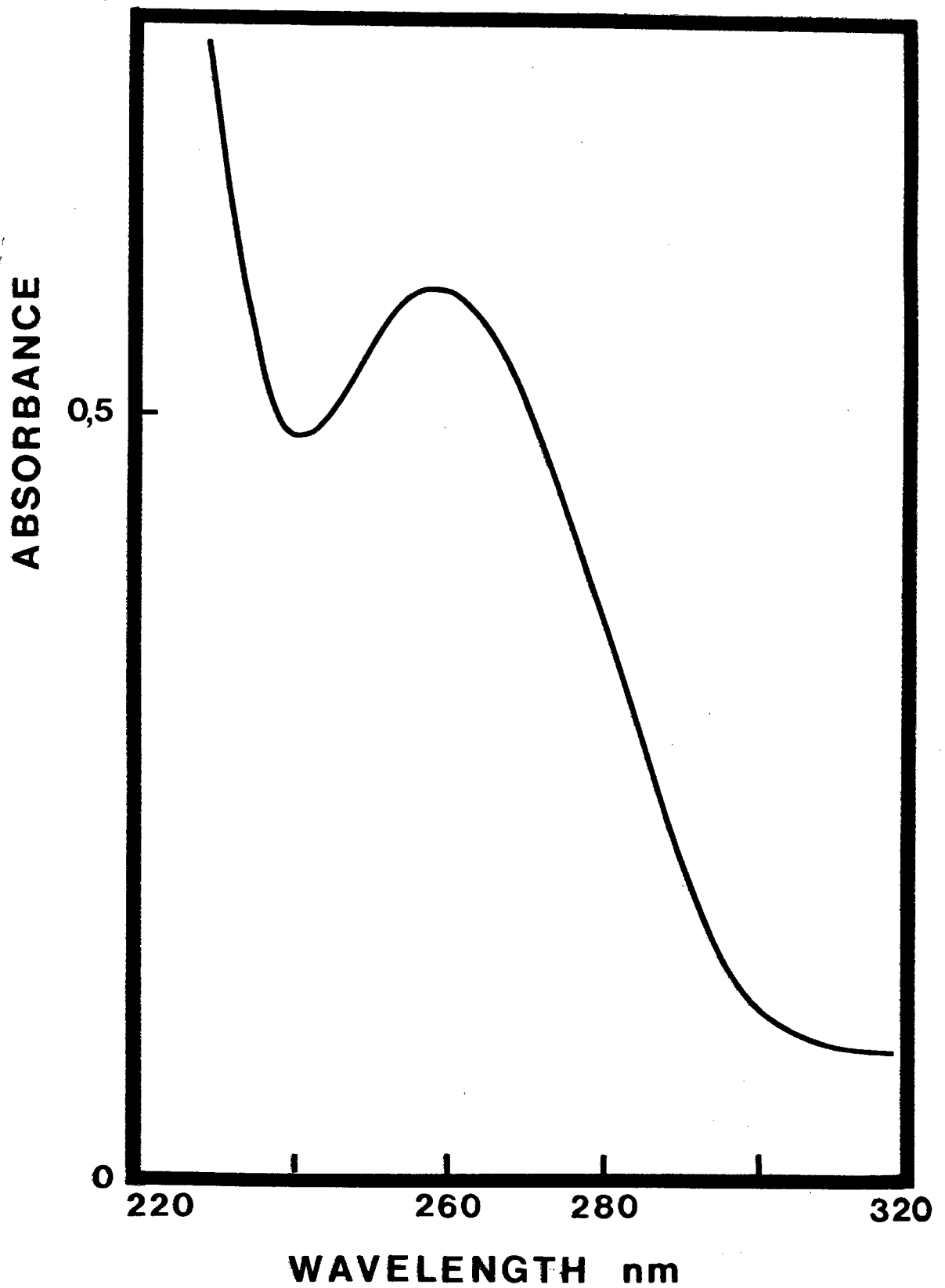


Figure 3 : The UV absorbance profile of MDMV-Kru isolate in 0,05M borate pH 8,1 after 1 cycle of differential ultracentrifugation.

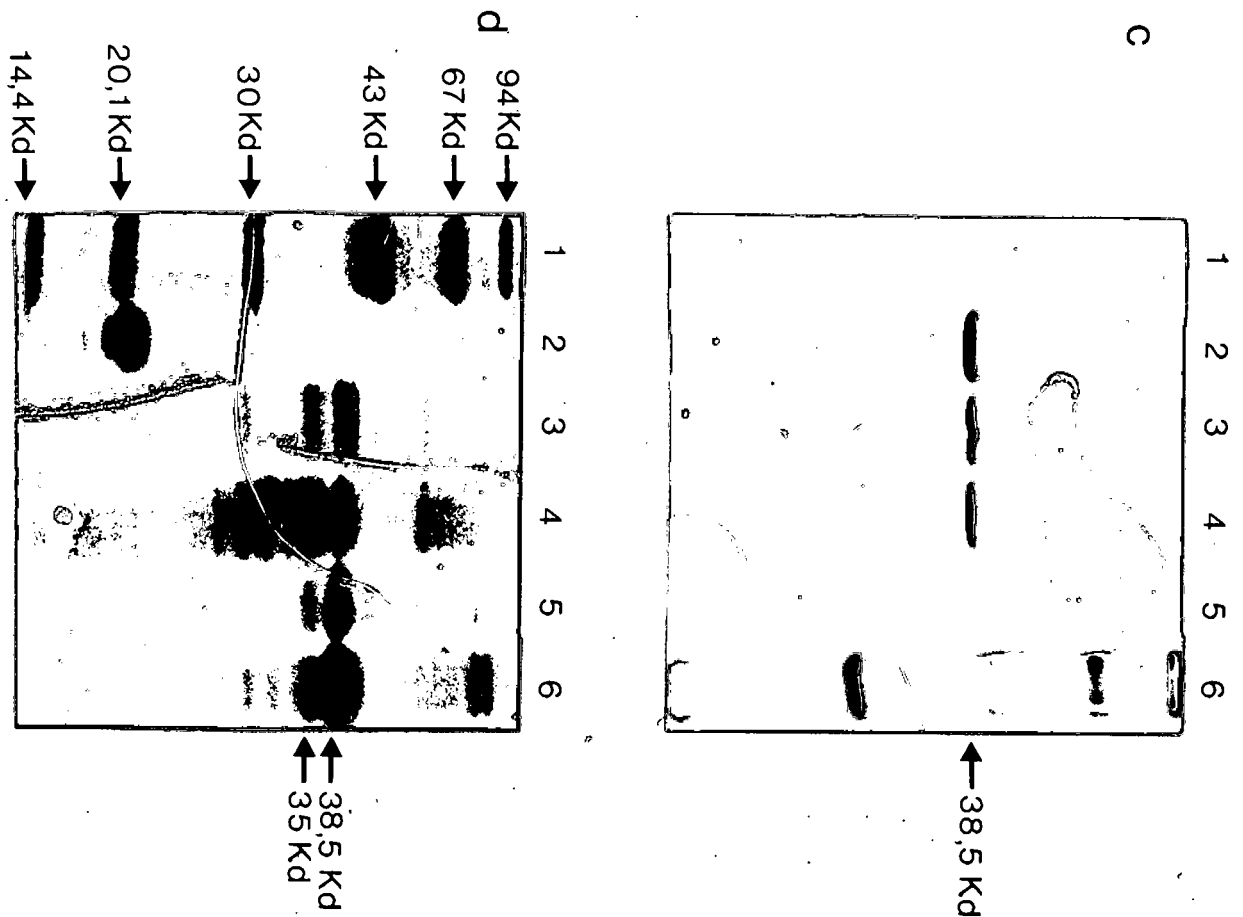
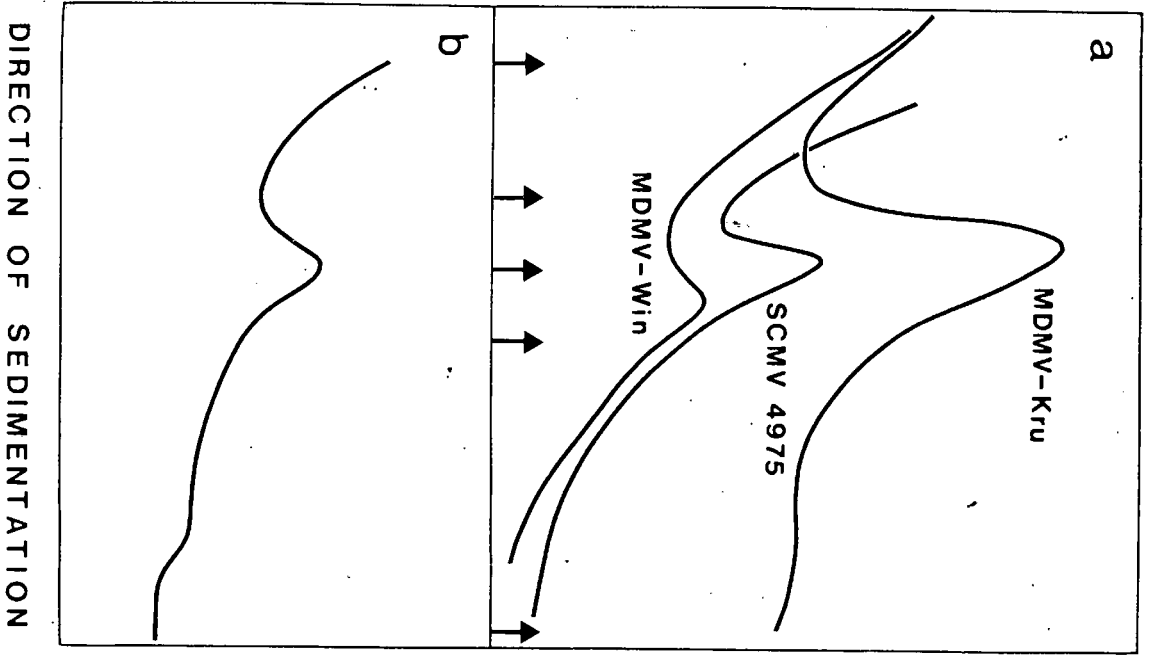
d. Rate zonal centrifugation

For further purification, virus was centrifuged after differential ultracentrifugation through a sucrose gradient (for details see III C4.b) to separate virus particles and residual plant protein. The sucrose gradient purified-virus was used as immunogen for the production of antisera in rabbits.

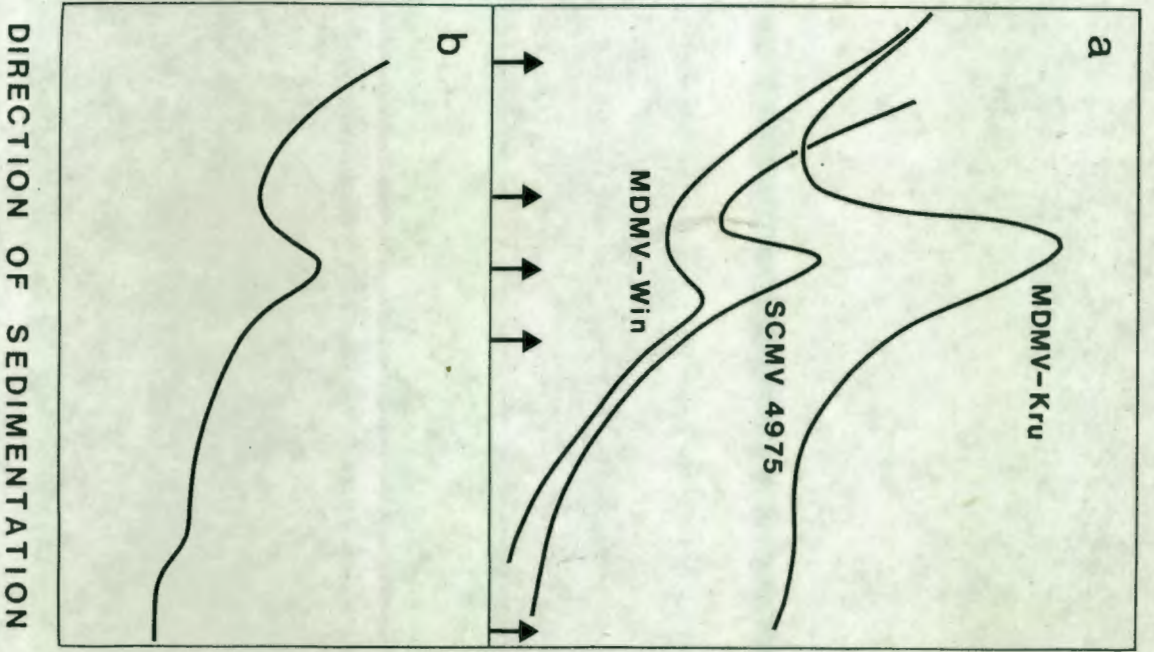
After rate zonal centrifugation, the tube contents were fractionated in an ISCO Model 604 fractionator and UA-5 absorbance unit and chart recorder. A single peak was recorded for each isolate. This corresponds to the opalescent band observed at 2,5 - 3cm below the meniscus in the tube. The U.V. absorbance profiles at 254mm of MDMV-Kru, SCMV 4975 and MDMV-Win are shown in Fig. 4a. The absorbance ratio,  $A_{260}/A_{280}$  for MDMV-Kru isolate was 1,25.

In a few cases, a second smaller peak was observed below the main fraction, Fig. 4b. The main fraction and the smaller fraction, lower down in the tube were separated, dialysed and inoculated onto maize plants. Both fractions were infective producing mosaic symptoms. The second peak may correspond to dimers or aggregates of virus particles. Examination under the electron microscope showed the presence of filamentous particles.

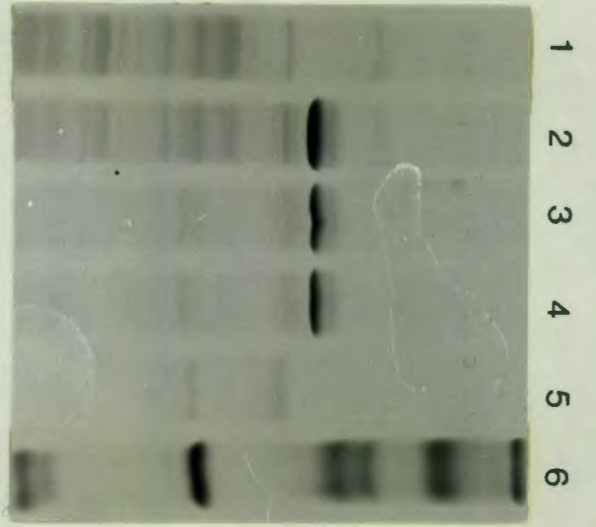
ABSORBANCE AT 254nm



ABSORBANCE AT 254nm



c



d

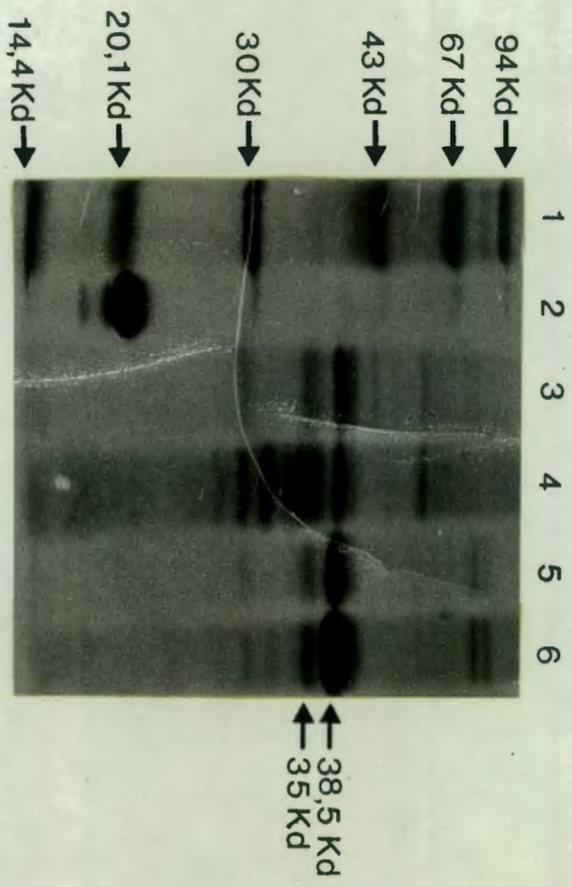


Figure 4 :

- (a) A typical UV absorbance profile at 254nm obtained after rate zonal centrifugation of MDMV-Kru, SCMV 4975 and MDMV-Win. The tube contents were scanned through an ISCO Model 604 fractionator.
- (b) The UV absorbance profile at 254nm of MDMV-Kru showing the 2 peaks obtained, the main fraction (large peak) and occasionally the faster sedimenting minor fraction (small peak).
- (c) Electrophoresis on SDS-polyacrylamide gel of MDMV-Bar purified by rate zonal centrifugation. The samples were taken from different depths of the tube after centrifugation.

Lane 1 = Fraction collected near the meniscus  
2 = Fraction collected just above the opalescent band  
3 = Fraction collected below the opalescent band  
4 = Opalescent band  
5 = Pellet at the bottom of the tube  
6 = MW protein markers, phosphorylase b 94Kd, bovine serum albumin 67Kd, ovalbumin 43Kd, carbonic anhydrase 30Kd, soybean trypsin inhibitor 20,1Kd

- (d) Electrophoresis on SDS-polyacrylamide gels of MDMV-Kru partially purified after 1 cycle of differential ultracentrifugation and after rate zonal centrifugation.

Lane 1 = MW protein markers, phosphorylase b 94Kd, bovine serum albumin 67Kd, ovalbumin 43Kd, carbonic anhydrase 30Kd, soybean trypsin inhibitor 20,1Kd,  $\alpha$ -lactalbumin 14,4Kd  
2 = Brome mosaic virus (BMV) 20,1Kd  
3 = MDMV-Kru after differential ultracentrifugation  
4 = MDMV-Kru after differential ultracentrifugation and using extraction buffer without additives, EDTA and Na<sub>2</sub>SO<sub>3</sub>  
5 = MDMV-Kru, opalescent band after rate zonal centrifugation  
6 = MDMV-Kru fraction corresponding to the minor peak in the UV absorbance profile at 254nm (Fig. b) after rate zonal centrifugation

A further test was done on the rate zonal purified preparations by electrophoresis of samples on SDS-polyacrylamide gels (for details on electrophoresis see III.G). The contents of the tube with MDMV-Bar isolate was sampled at different depths with a hand held syringe after rate zonal centrifugation. The samples were collected near the meniscus, just above the opalescent band, at the opalescent band and just below the band. Samples were dialysed, centrifuged at H.S. and dissociated for electrophoresis on gels. The results for MDMV-Bar sample are shown in Fig. 4c. The host proteins were above the opalescent bands, as shown in Fig. 4c, Lanes 1 and 2. There was one major component found in the rate zonal separated samples at 38,5Kd. The approximate distance from the meniscus at which the samples were taken are shown by the arrows in Fig. 4a.

MDMV-Kru isolate was centrifuged through a sucrose gradient and the tube contents were fractionated through an ISCO fractionator. The two fractions corresponding to the main fraction and the smaller faster sedimenting fraction Fig. 4b, were dialysed, centrifuged and electrophoresed. Lane 6, Fig. 4d shows the faster sedimenting sample and Lane 5 shows the peak corresponding to the opalescent band. The molecular weight of both samples was identical at 38,5Kd. A second, weaker protein band was present in both samples

at the 35,5Kd position. The faster sedimenting fraction obtained after rate zonal centrifugation was not further investigated as this was probably aggregates of the virus.

Samples of MDMV-Kru obtained after 1 cycle of differential ultracentrifugation are shown in Fig. 4d Lanes 3 and 4. One sample was purified with additives (EDTA and  $\text{Na}_2\text{SO}_3$ ) in the extraction buffer (Lane 3) and the other without (Lane 4). The major polypeptide band is at 38,5Kd position for both samples, with a larger number of lower molecular weight polypeptides present in the sample purified without EDTA and  $\text{Na}_2\text{SO}_3$ . These polypeptides are probably broken down viral protein. (See IEB assay, section IV A 10 b). More degradation of virus protein occurred during the purification procedure when EDTA and  $\text{Na}_2\text{SO}_3$  were not used.

##### 5. ZONE ELECTROPHORESIS

Alternately virus preparations were further purified after differential ultracentrifugation, by electrophoresis in a sucrose gradient (see Section III 4.a). Pure virus preparations, free from host protein contaminants, were used as immunogens in the production of rabbit antisera. A comparison of the two methods, zone electrophoresis and zonal centrifugation was not done.

Table 7 : The  $R\emptyset$  values of the MDMV isolates as determined by zone electrophoresis

| <u>Isolate</u> | <u>Range of <math>R\emptyset</math> values obtained</u> | <u>No. of trial runs</u> | <u>Average <math>R\emptyset</math> value</u> |
|----------------|---------------------------------------------------------|--------------------------|----------------------------------------------|
| MDMV-Bar       | 0,16 - 0,164                                            | 2                        | 0,16 $\pm$ 0,002                             |
| MDMV-Kru       | 0,15 - 0,36                                             | 8                        | 0,19 <sup>1</sup> $\pm$ 0,07                 |
| MDMV-Win       | 0,13 - 0,17                                             | 3                        | 0,15 $\pm$ 0,02                              |
| SCMV 4975      | 0,16 - 0,20                                             | 3                        | 0,18 $\pm$ 0,02                              |
| Host pigment   | 0,65 - 0,93                                             | 14                       | 0,76 $\pm$ 0,07                              |
| SCMV           |                                                         |                          | 0,21 <sup>2</sup>                            |

1. The opalescent band at this  $R\emptyset$  value was isolated and centrifuged at H.S. This was infective on maize plants, showing mosaic symptoms 7 days post inoculation. The absorbance ratio A260/A280 was 1,18, an average of 2 readings.

2. This value obtained from von Wechmar (1967).

## 6. ANTISERA

Antisera were raised against the MDMV- and SCMV- isolates as described in section III D. Preparations obtained after rate zonal centrifugation or after zone electrophoresis in a sucrose gradient were injected with an equal volume of Freund's incomplete adjuvant, intramuscularly into rabbits. Rabbits were injected once a week for 2 to 4 weeks and then given injections after 6 - 8 weeks (see section III D.1). Rabbits were bled weekly through a marginal ear vein.

A typical antiviral immune response over a period of 27 weeks is shown in Fig. 5. Rate zonal purified MDMV-Kru was injected into a rabbit at intervals as shown by the arrows in Fig. 5. A second rise in antibody titre was observed after 100 days (Fig. 5). This is due to the booster effect of the repeated injections. The titres of the antiserum bleedings were determined by the tube precipitin test.

The MDMV and SCMV isolates were tested in homologous and heterologous reactions with their respective antisera by the tube precipitin and microprecipitin tests. The titres obtained are shown in Table 8.

RECIPROCAL OF ANTIBODY TITRE

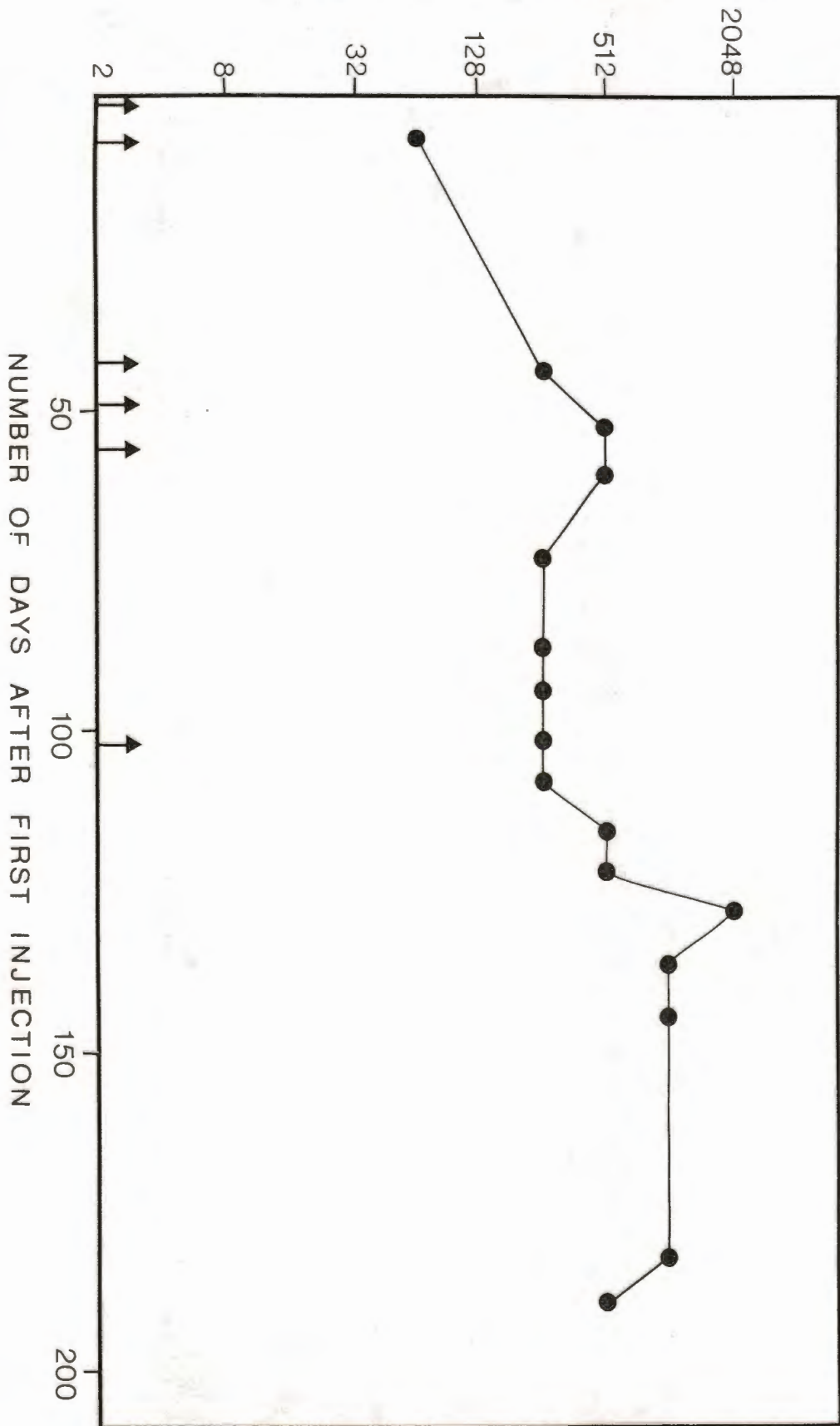


Figure 5 :

The immune response curve of a rabbit immunized with MDMV-Kru antigen. The antiserum titres were determined by the tube precipitin or microprecipitin tests. The arrows indicate times at which the rabbit was injected.

Table 8 : The homologous and heterologous reactions of MDMV and SCMV isolates with their respective antisera as determined by the tube precipitin and microprecipitin tests.

| Antibody                                         | Antigen          |          |          |          |
|--------------------------------------------------|------------------|----------|----------|----------|
|                                                  | SCMV 4975        | MDMV-Win | MDMV-Kru | MDMV-Bar |
| anti-SCMV 4975-1(ZE) <sup>1</sup>                | 256 <sup>4</sup> | 256      | 256      | 128      |
| anti-SCMV 4975<br>-2 (Rate Z)                    | 256              | 128      | 128      | 128      |
| anti-MDMV-Win -1(ZE)                             | 512              | 1024     | 512      | 512      |
| anti-MDMV-Win-2 (Rate Z)                         | 32               | 64       | 32       | 32       |
| anti-MDMV-Kru<br>-1(Rate Z) (early) <sup>2</sup> | 64               | 256      | 512      | 128      |
| anti-MDMV-Kru-1(late) <sup>3</sup>               | 512              | 1024     | 1024     | 512      |
| anti-MDMV-Kru-2 (ZE)                             | 512              | 2048     | 2048     | 512      |
| anti-MDMV-Bar-1 (Rate Z)                         | 128              | 256      | 256      | 256      |
| anti-MDMV-Bar-2 (ZE)                             | 4                | 32       | 32       | 64       |

1 = Antiserum obtained by immunization with virus purified by zone electrophoresis in a sucrose gradient (ZE) or by rate zonal centrifugation (Rate Z)

2 = An early bleeding of the serum, 59 days after 1st injection

3 = A later bleeding of the serum, 136 days after 1st injection

4 = The reciprocal of the antibody titre

In nearly all cases, the heterologous titres were lower than the homologous reactions. All four isolates are

serologically related as shown by the positive reactions obtained in Table 8. The isolates are closely related, but not identical as shown by the above test.

Host absorbed antisera were selected for further immunological tests, and the gamma-globulin fractions were purified by ammonium sulphate precipitation as described in section III.D.3. The titres of these antisera are given in Table 9.

Table 9 : The titres of antisera used in further immunological tests.

| <u>Antiserum</u> | <u>Immunogen</u>               | <u>Titre</u>                        |
|------------------|--------------------------------|-------------------------------------|
| anti-SCMV 4975-1 | SCMV 4975 (ZE) <sup>1</sup>    | 256 <sup>3</sup> (137) <sup>4</sup> |
| anti-MDMV-Win-1  | MDMV-Win (ZE)                  | 1024 (120)                          |
| anti-MDMV-Kru-1  | MDMV-Kru (Rate Z) <sup>2</sup> | 1024 (136)                          |
| anti-MDMV-Bar-1  | MDMV-Bar (Rate Z)              | 256 (122)                           |

1 = ZE refers to virus injected after zone electrophoresis in a sucrose gradient

2 = Rate Z refers to virus injected after rate zonal centrifugation

3 = Reciprocal of the titre determined by tube precipitin or microprecipitin tests

4 = The number of days after the 1st injection when bleeding was obtained

The MDMV-Kru isolate was tested by the tube precipitin test with SCMV-antisera obtained from the antiserum

collection, Microbiology Dept., UCT. The results are shown in Table 10.

Table 10 : The reactions of MDMV-Kru isolate with anti-SCMV and MDMV antisera in the tube precipitin test

| <u>Antiserum</u>   | <u>Reciprocal of titre</u> |
|--------------------|----------------------------|
| anti-SCMV Martin-1 | 128                        |
| anti-SCMV Martin-2 | 128                        |
| anti-SCMV 376      | 128                        |
| anti-MDMV-Kru-1    | 256                        |

## 7. BIOPHYSICAL STUDIES

### a. The buoyant density in CsCl

The opalescent band obtained after rate zonal centrifugation was dialysed against 0,1M  $KPO_4$  pH 7,0 before centrifuging to equilibrium in a self-generating CsCl gradient with a starting concentration of CsCl at  $1,36g/cm^3$ . (For details, see III.C.4(c)). The buoyant densities of the four isolates were calculated from the refractive indices. Table 11 lists the values obtained and Fig. 6 illustrates a typical curve obtained for MDMV-Bar.

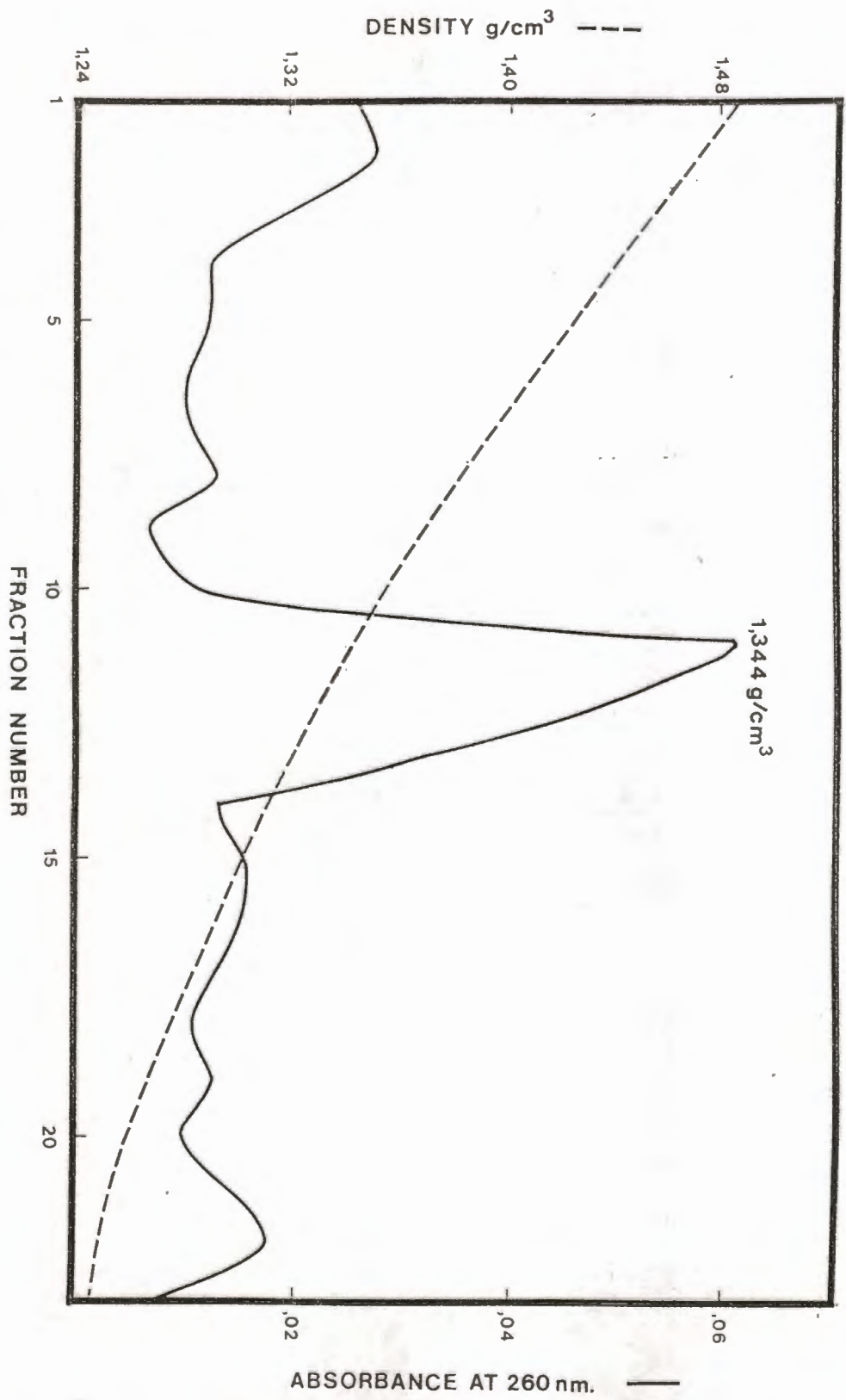


Figure 6 : A typical profile obtained for MDMV-Bar after isopycnic density gradient centrifugation in CsCl. The calculated density was 1,344g/cm<sup>3</sup>.

Table 11 : The buoyant density in CsCl of the MDMV  
and SCMV isolates

| <u>Isolate</u> | <u>Buoyant density<br/>(g/cm<sup>3</sup>)</u> | <u>Absorbance ratio<br/>A260/A280</u>         |
|----------------|-----------------------------------------------|-----------------------------------------------|
| MDMV-Bar       | 1,3444                                        | 1,36                                          |
| MDMV-Kru       | 1,3374                                        | 1,175                                         |
| MDMV-Win       | 1,3391                                        | 1,23                                          |
| SCMV 4975      | 1,3475                                        | 1,22                                          |
| MDMV-A         | 1,3421 <sup>1</sup>                           | 1,13 - 1,16 <sup>1</sup>                      |
| MDMV-B         | 1,3427 <sup>1</sup>                           | 1,14-1,17 <sup>1</sup> ,<br>1,26 <sup>2</sup> |
| SCMV-D         | 1,3327 <sup>3</sup>                           | -                                             |

1 - Data from Tosic and Ford (1974)

2 - Data from Langenberg (1973)

3 - Data from Pirone (1972)

b. Determination of protein MW by SDS-PAGE

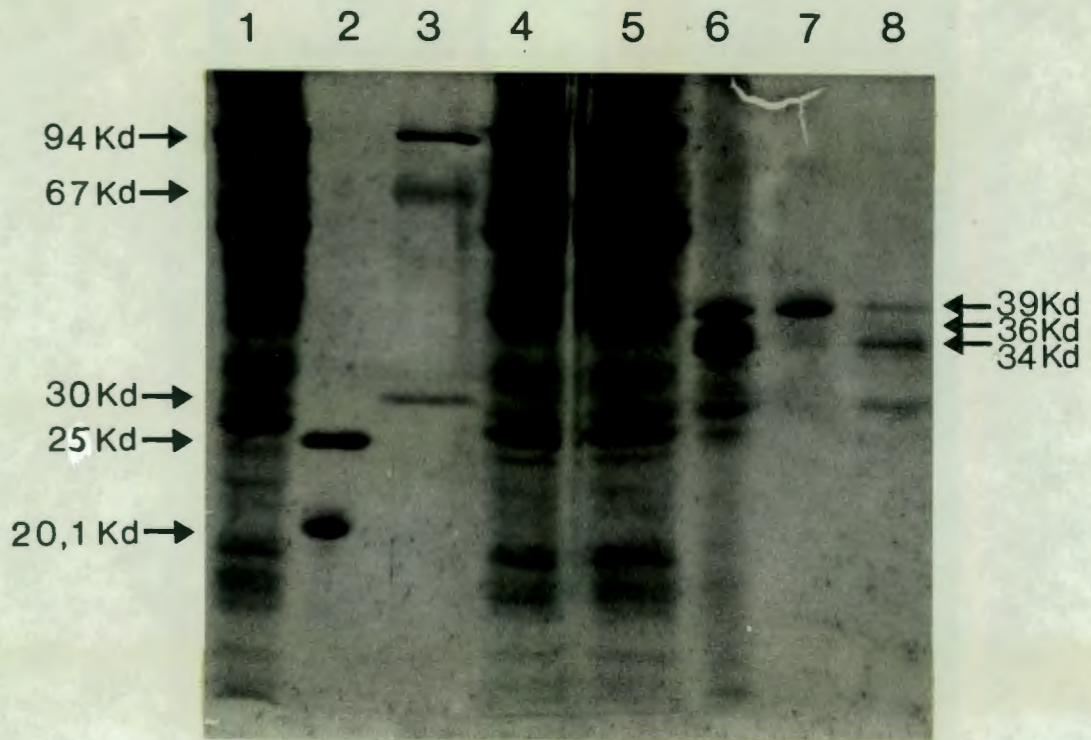
The various isolates were electrophoresed in SDS-polyacrylamide gels consisting of a 4,5% stacking gel and a 12,5% resolving gel (see Section III.G.1). The molecular weight of the dissociated virus protein was estimated by comparison to a standard curve obtained by electrophoresis of proteins of known molecular weights (low molecular weight standard proteins, Pharmacia). Virus isolates were dissociated after 1 cycle of differential

ultracentrifugation, after zonal centrifugation in a sucrose gradient or after isopycnic density gradient centrifugation in CsCl.

The estimated molecular weights of the virus protein of the isolates was in the range 37,5Kd to 39Kd, with a series of lower molecular weight polypeptides of 30Kd to 36Kd. No significant difference in molecular weight of the isolates were observed. An example of a gel is shown in Fig. 7. The proteins were electrophoresed at 10mA overnight and the gel was stained with Coomassie blue.

The gel was used to calculate the molecular weights and used for IEB assay for comparison of the isolates. The actual gel of Fig. 7 was electroblotted onto nitrocellulose paper (see Section III.G.2) and this may be the reason that some protein bands are less strong than in other Coomassie stained gels. The electroblot of this gel appears in Fig. 14c (Section IV.A.9(b)).

The major band appearing in the samples (Fig. 7, lanes 6, 7 and 8) of MDMV-Bar, MDMV-Kru and MDMV-Win respectively, is at the 39Kd position. This band is not present in the uninfected maize sample shown in lane 1, but is present in the MDMV-Win and -Kru infected maize sap samples in lanes 4 and 5 respectively. The electrophoretic profile of the MDMV-Bar isolate (lane 6) shows



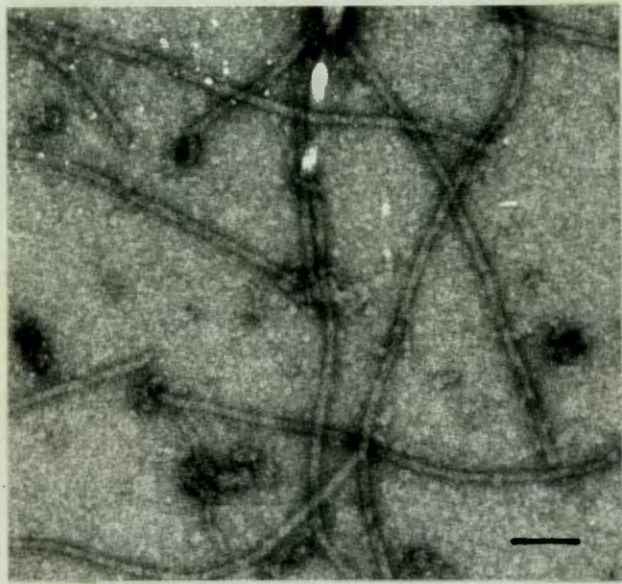
**Figure 7 :** Coomassie stained SDS-polyacrylamide gel.  
 Electrophoresis was done at 10mA overnight.  
 Lane 1 = Uninoculated KEP-Witplat maize  
 Lane 2 = Barley stripe mosaic virus protein  
 marker, 25Kd and Brome mosaic virus  
 protein, 20,1Kd  
 Lane 3 = Protein molecular weight markers  
 (Pharmacia)  
 Lane 4 = MDMV-Win infected maize sap  
 Lane 5 = MDMV-Kru infected maize sap  
 Lane 6 = MDMV-Bar (after differential centrifugation)  
 Lane 7 = MDMV-Kru (CsCl purified)  
 Lane 8 = MDMV-Win (rate zonal purified virus)

This photograph does not show SCMV 4975 but  
 calculations from other gels showed the major MW  
 band did not differ from the bands for the other  
 isolates.

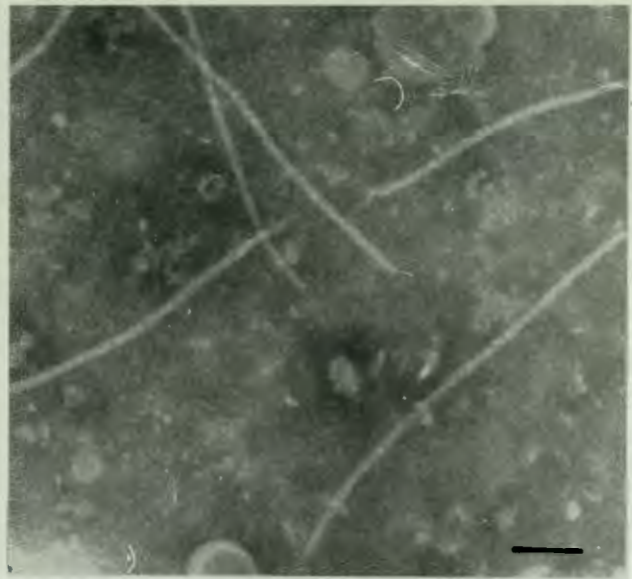
a number of polypeptides, with four major bands. This shows that the processing of the virus caused a breakdown of the protein. After rate zonal purification less polypeptides are apparent (lane 8, rate zonal purified MDMV-Win) and after isopycnic centrifugation in CsCl, only one major band is present. Fig. 7, lane 7 shows the CsCl purified MDMV-Kru isolate. (Also see Fig. 4d, Section IV.A.4(d)).

## 8. ELECTRON MICROSCOPY

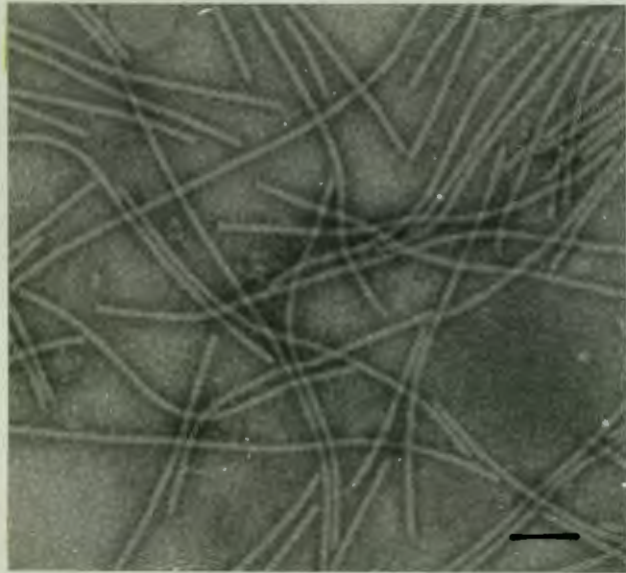
Fig. 8a shows a typical electron micrograph of a leaf crush sample of one of the isolates, SCMV 4975. Filamentous particles of MDMV-Kru ranging from approximately 622nm to 744nm were observed. The width of the particles were approximately 12 - 13nm. A preparation of MDMV-Kru after one cycle of HS centrifugation is shown in Fig. 8b. Host particles were still present in this partially purified preparation. Fig. 8c is an electron micrograph of a purified preparation of MDMV-Kru after rate zonal centrifugation, showing the presence of filamentous particles. A sample of the 2nd faster sedimenting fraction observed occasionally after rate zonal centrifugation (see Section IV.A.4(d)), when examined revealed the presence of filamentous particles (see Fig. 8d). The purified preparation of MDMV-Kru obtained after isopycnic centrifugation in CsCl is shown in Fig. 8e.



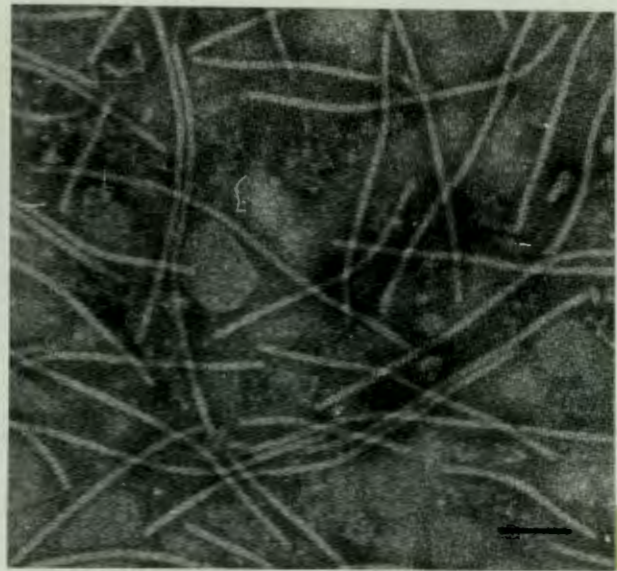
a



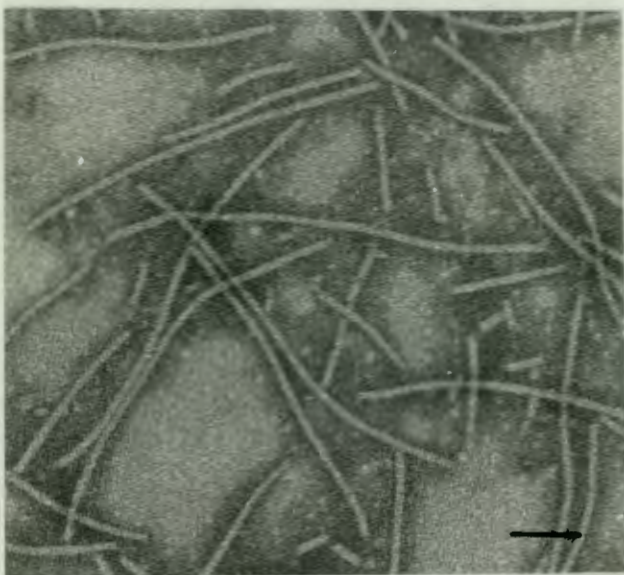
b



c



d



e

Figure 8 :

Electron micrographs of the typical filamentous particles found in preparations of MDMV and SCMV at different stages of purification.

- a. A leaf squash of SCMV 4975 infected maize.
- b. Partially purified MDMV-Kru after 1 cycle of differential ultracentrifugation.
- c. MDMV-Kru after rate zonal centrifugation in 20 - 50% sucrose gradient.
- d. The 2nd faster sedimenting fraction sometimes obtained in rate zonally purified preparations of MDMV-Kru (see section IV.A.4(d)).
- e. MDMV-Kru preparation after isopycnic gradient centrifugation in CsCl.

Negative stains were 2% uranylacetate (a,d) and 2% PTA (b,c,e). Magnification is 90 000X. Bars represent 100nm.

## 9. RELATIONSHIP STUDIES

Three different techniques were used to establish the relationship of the maize isolates. These were (a) ELISA, (b) IEB and (c) ISEM.

### (a) ELISA

The ELISA technique was one of the methods used for studying the relationship of the MDMV and SCMV isolates. It was of interest to compare the isolates serologically, to determine whether there were any differences, as the isolates were obtained from different areas.

#### (i) Calibration of antibody

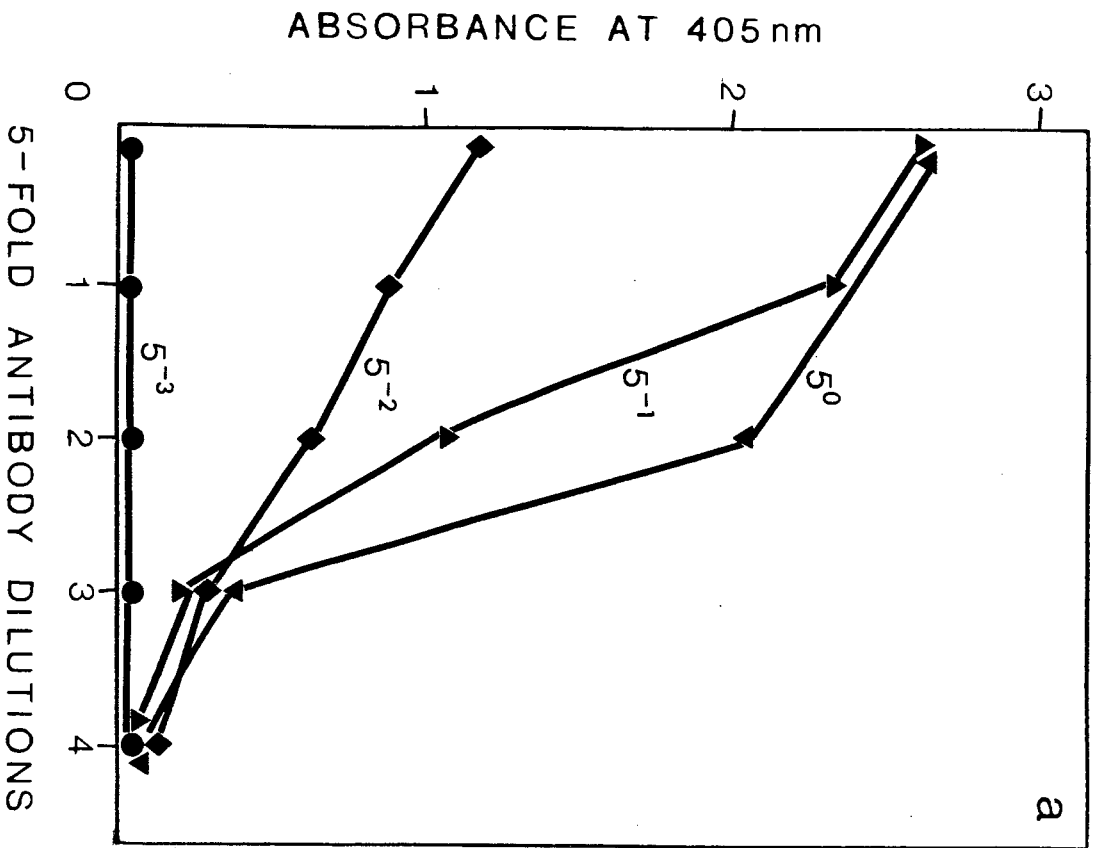
The optimal concentrations of antiserum and antigen were determined for use in the sandwich and indirect ELISA tests (see Section III.4(d)).

An example of the curves obtained for a grid titration using the indirect ELISA test is shown in Fig. 9.

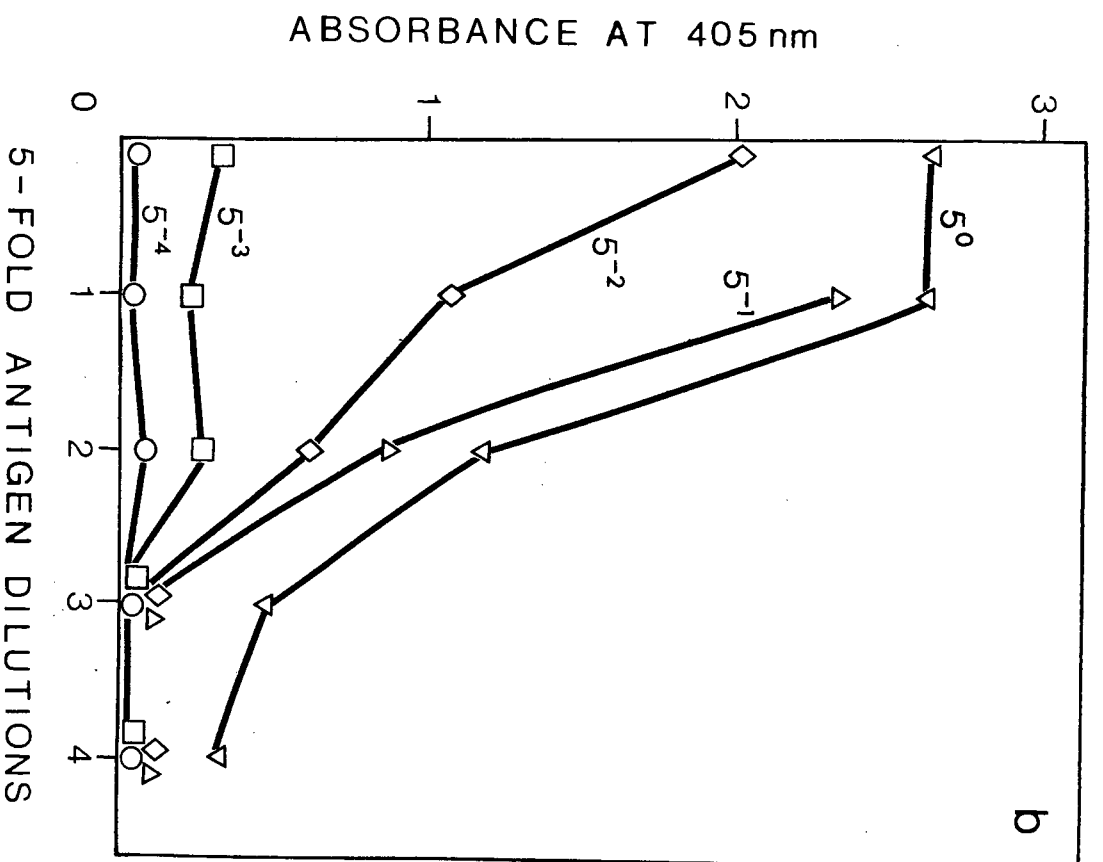
5-fold dilutions of Ag(MDMV-Kru) diluted in PBS pH 7,0 with a starting concentration of 0,2mg/ml were used.

Antiserum was diluted 5-fold in PBS-Tween-BSA, at a starting concentration of 1/100. From Fig. 9a, it can be seen that the best curve obtained for differing antibody concentrations, was at 0,04mg/ml MDMV-Kru.

At increasing dilutions of Ag, Fig. 9b, the optimum concentration of anti-MDMV-Kru-1 IgG was at 1/100



(a) The reaction of anti-MDMV-Kru-1 with MDMV-Kru. Antibody was diluted 5-fold at a starting dilution of 1/100. MDMV-Kru was diluted 5-fold at a starting concentration of 0,2mg/ml.  $5^0 = 0,2\text{mg/ml}$ .



(b) The reciprocal of (a) above. The starting concentration of antigen and antibody as above  $5^0 = 1/100$  dilution of anti-MDMV-Kru IgG.

Figure 9 : Calibration of antisera (IgG) by indirect ELISA technique.

dilution. The optimal dilutions of the antisera used for the relationship studies in the indirect ELISA test, are given below in Table 12.

Table 12 : The optimal dilutions of various antisera raised against different isolates used in the indirect ELISA tests

| <u>Antibody</u>  | <u>Dilution</u> |
|------------------|-----------------|
| anti-MDMV-Bar-1  | 1/100           |
| anti-MDMV-Kru-1  | 1/100           |
| anti-MDMV-Win-1  | 1/300           |
| anti-SCMV 4975-1 | 1/100           |

The optimal dilutions of coating antibody and alkaline phosphatase conjugated IgG was determined for use in the sandwich ELISA test. The calibration curves obtained for anti-SCMV 4975 coating antibody is shown in Fig. 10. The optimum dilution of coating antibody was at 1/500 dilution. The optimum dilution of conjugated IgG was at 1/400 dilution. The optimal dilutions of coating antibody and conjugates of the three antisera used in the sandwich ELISA test are given in Table 13 which follows:

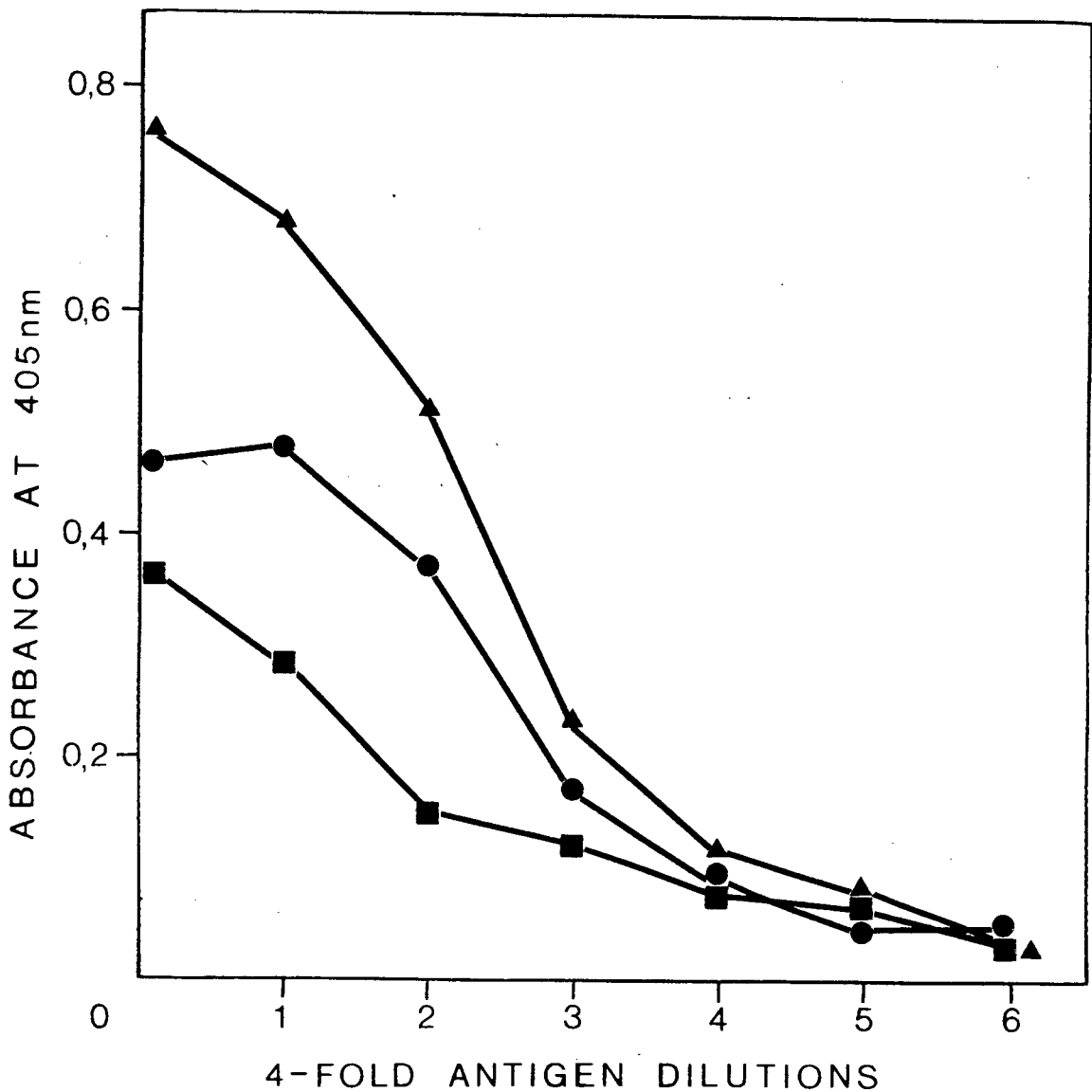


Figure 10 : Reaction of SCMV 4975 with anti-SCMV 4975 IgG in the sandwich ELISA.

SCMV 4975 was diluted 4-fold at a starting concentration of 0,2mg/ml. Alkaline phosphatase-IgG conjugate was diluted at 1/400. The coating antibody was used at dilutions of 1/50, 1/100, 1/500.

■ represents the reaction at 1/50 dilution of coating antibody

● at 1/100, and

▲ at 1/500.

Table 13 : The optimal dilutions of coating antibody  
and alkaline phosphatase conjugated IgG  
used for the sandwich ELISA tests

| <u>Coating antibody</u> | <u>Dilution</u> |
|-------------------------|-----------------|
| anti-MDMV-Kru-1         | 1/400           |
| anti-MDMV-Win-1         | 1/300           |
| anti-SCMV 4975-1        | 1/400           |
| <br>                    |                 |
| <u>Conjugated IgG</u>   |                 |
| anti-MDMV-Kru-1         | 1/500           |
| anti-MDMV-Win-1         | 1/300           |
| anti-SCMV 4975-1        | 1/500           |

(ii) Relationship studies of the MDMV isolates

The three MDMV isolates and the SCMV isolate are related with slight differences among them, as determined by the indirect ELISA test. Fig. 11 shows the reactions of MDMV-Kru (Fig. 11a) MDMV-Bar (Fig. 11b) MDMV-Win (Fig. 11c) and SCMV 4975 (Fig. 11d) to their respective homologous antisera. Simultaneously each Ag was also tested to the respective heterologous antisera. The concentrations of Ag was at 0,04mg/ml and the Ab was diluted 5-fold at a starting dilution of 1/100. MDMV-Kru appears to pick up differences when reacted with the four antisera (Fig. 11a) and MDMV-Bar shows these differences to a lesser degree (Fig. 11b). In contrast MDMV-Win and SCMV 4975 do not show these differences (Fig. 11c and d).

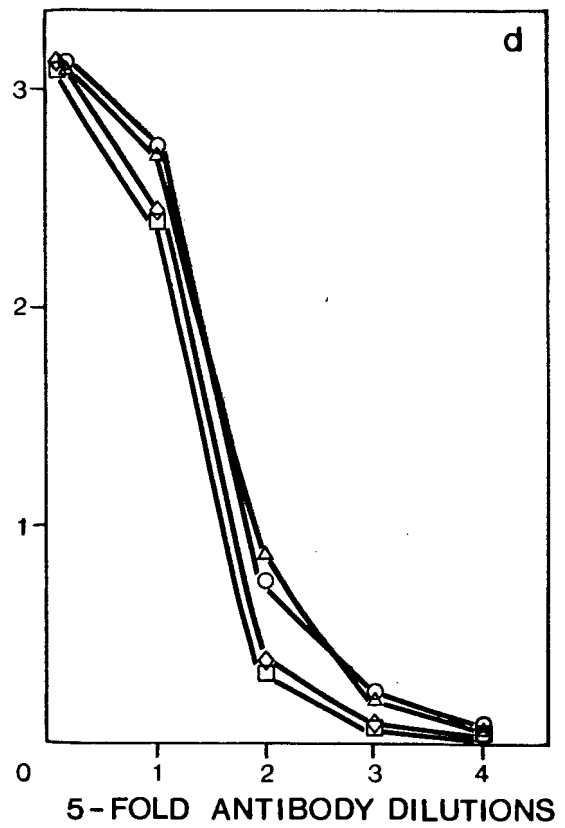
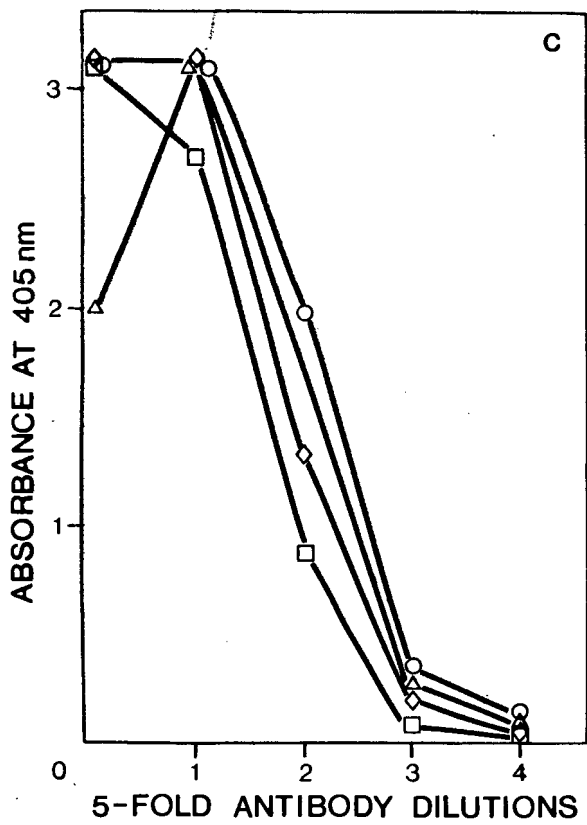
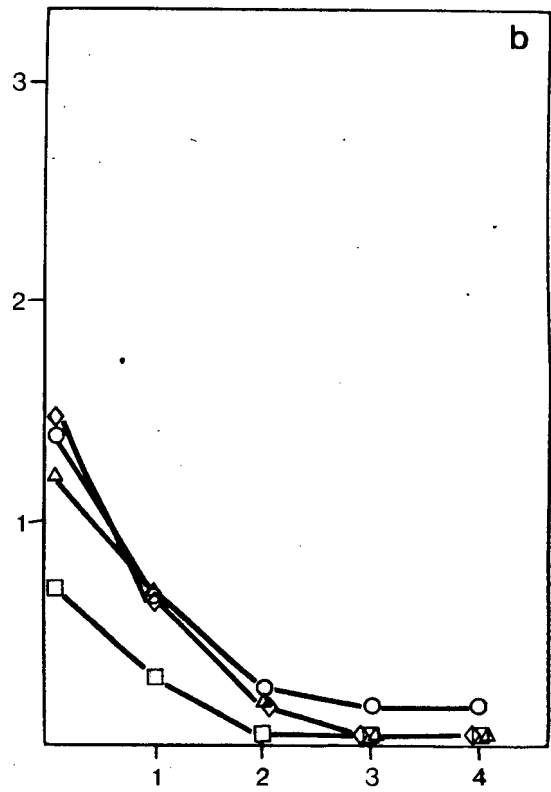
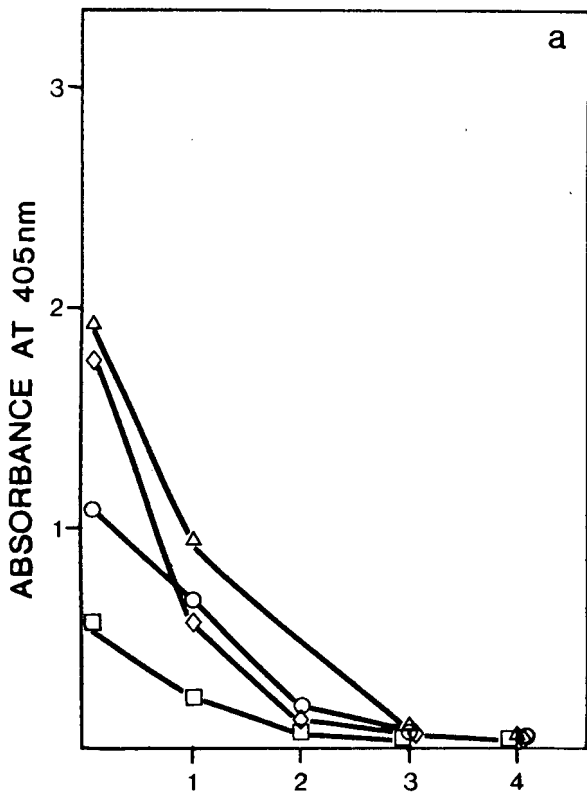
Figure 11 :

An indirect ELISA test showing the relationship of:

- a) MDMV-Kru
- b) MDMV-Bar
- c) MDMV-Win
- d) SCMV 4975

The antigen concentration was 0,04mg/ml. The MDMV- and SCMV- IgG's were diluted 5-fold at a starting dilution of 1/100. The alkaline phosphatase-conjugated GAR IgG was used at 1/1500 dilution.

- △ = anti-MDMV-Kru-1
- = anti-MDMV-Bar-1
- ◇ = anti-MDMV-Win-1
- = anti-SCMV 4975-1



From these results it appears that MDMV-Kru recognizes differences not reflected in the other Ag-Ab combinations. It is also of interest to note that anti-MDMV-Bar-1 shows the least reaction in the Ag-Ab combinations. This may suggest that MDMV-Bar may be more distantly related to the other three isolates.

Another test, this time a sandwich ELISA, was performed to test the relationships of the isolates. MDMV-Win and SCMV 4975 were tested against anti-MDMV-Kru-1, anti-MDMV-Win-1 and anti SCMV 4975-1 sera in the sandwich ELISA test. From Fig. 12a it can be seen that there is very little difference in the reactions between the three antisera and MDMV-Win. Similarly, the reactions with SCMV 4975 and the same three antisera also show little difference (Fig. 12b). This seems to indicate that a close relationship exists between these two isolates. See Table 13 for coating Ab and alkaline phosphatase conjugated IgG dilutions used in the test.

The other Ag-Ab combinations were not tested in the sandwich ELISA test as, at this point in the investigation, seed transmitted MDMV was detected and it was feared that the seed transmitted isolate could have a strong influence on these relationship studies as all of the antisera were raised against virus isolates propagated in the K.E.P.-Witplat maize (see seed transmission, section IV.C.).

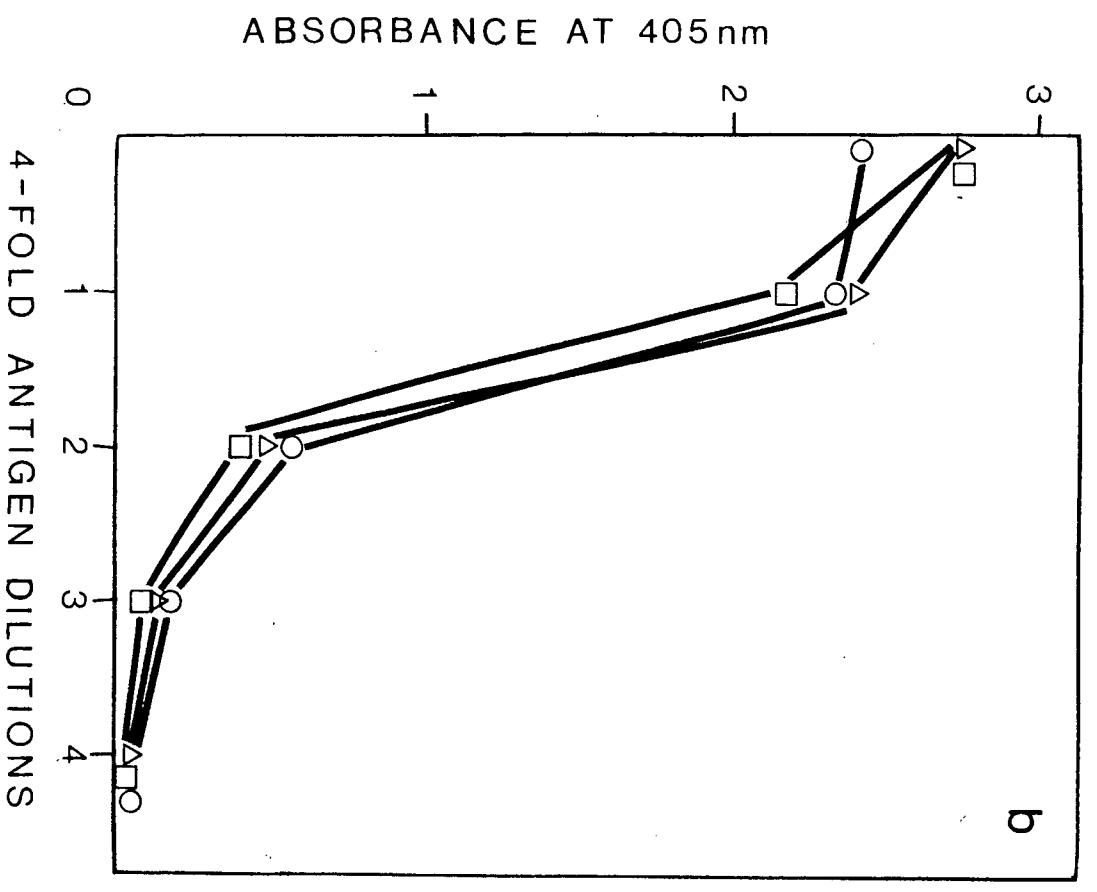
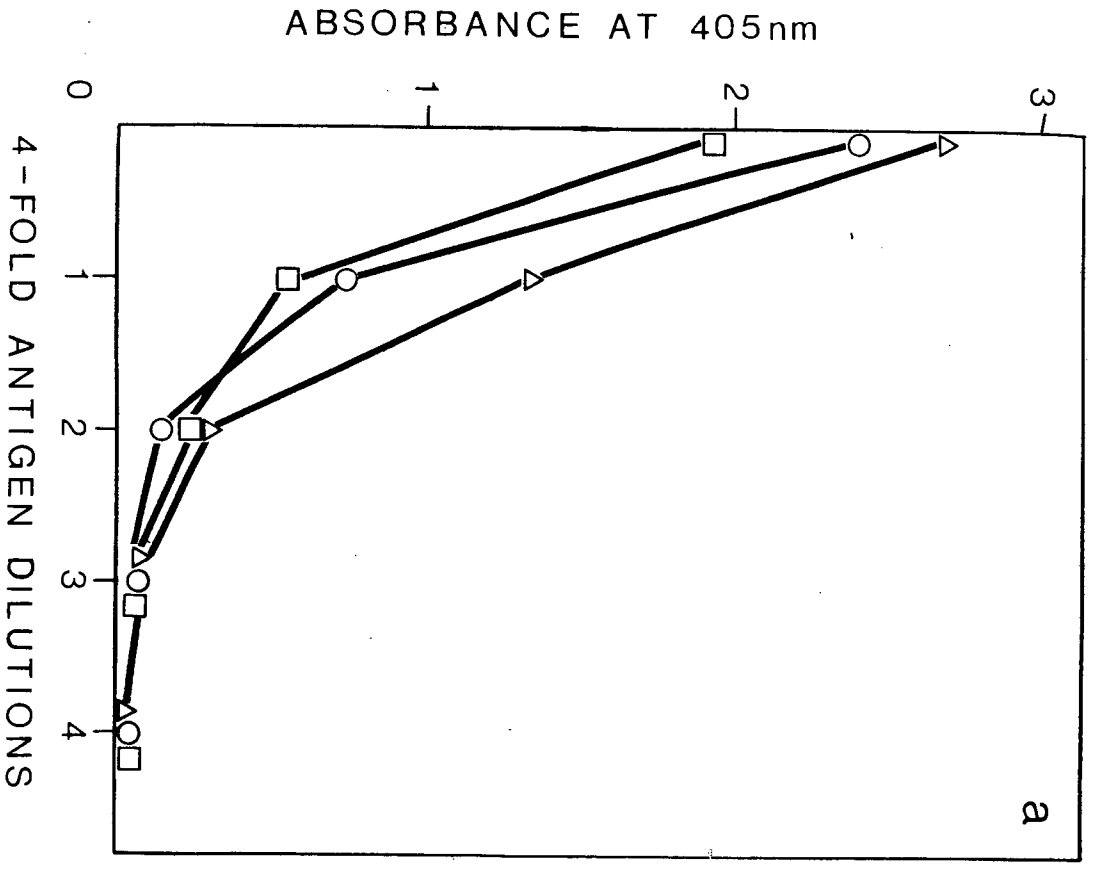


Figure 12 :

The reactions of MDMV-Win (a) and SCMV 4975 (b) against anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-SCMV 4975-1 antisera in the sandwich ELISA. The antigens were diluted 4-fold at a starting concentration of 0,2mg/ml.

Δ represents anti-SCMV 4975-1

□ represents anti-MDMV-Kru-1

○ represents anti-MDMV-Win-1

(iii) Relationship studies of the virus isolates with foreign MDMV antisera

It is known that there are two distinct groups of MDMV which are serologically different. These are the MDMV-A group or the Johnsongrass-infecting strains and the MDMV-B group or the strains that do not infect Johnsongrass. Louie and Knoke (1975) recorded six strains of MDMV, MDMV-A to F. (see section II). It was of interest to test the virus isolates under investigation against antisera to MDMV-A and MDMV-B to determine whether the isolates were related to these strains.

An indirect ELISA test was done to compare the virus isolates. The MDMV isolates and SCMV 4975 were tested with IgG preparations of antisera raised against MDMV-A and MDMV-B, which were kindly supplied by Prof. D.T. Gordon (Ohio State University, U.S.). The IgG preparations were used at  $\mu\text{g}/\text{ml}$  concentration. The antigens were diluted 4-fold with a starting concentration of  $0,5\text{mg}/\text{ml}$ . Fig. 13 shows that all four isolates react positively with anti-MDMV-B IgG, but react very slightly or not at all against anti-MDMV-A IgG (Fig. 13b). This indicates that the isolates are related to MDMV-B but not to MDMV-A.

The MDMV-Win and MDMV Seed-transmitted (MDMV-ST) (see Seed transmission, section IV.C.) isolates were tested against anti-MDMV-A and anti-MDMV-B IgG in the sandwich

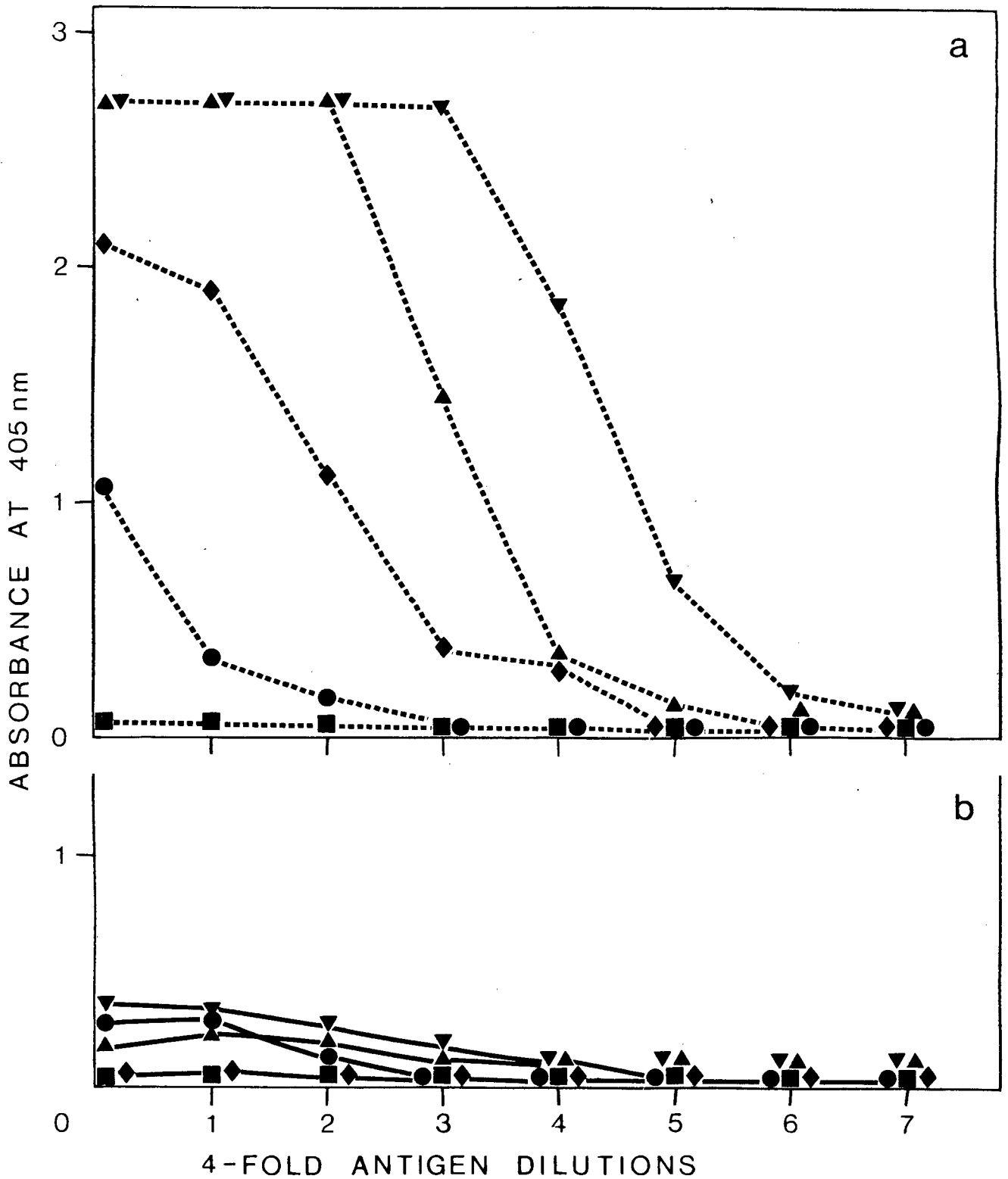


Figure 13 : The reaction curves of MDMV and SCMV isolates against anti-MDMV-B (.....) and -MDMV-A (—) IgG in the indirect ELISA. IgG was used at 1µg/ml concentration and the starting antigen concentration was 0,5mg/ml. The symbols represent the following antigens:

- ▼ = MDMV-Kru
- ▲ = MDMV-Win
- ◆ = SCMV 4975
- = MDMV-Bar
- = Uninfected maize control

ELISA test. The absorbances at 405nm that were obtained for the highest dilutions of Ag in this test are given in Table 14.

Table 14 : The absorbance at 405nm of antigens tested against anti-MDMV-A and anti-MDMV-B antisera in the sandwich ELISA test

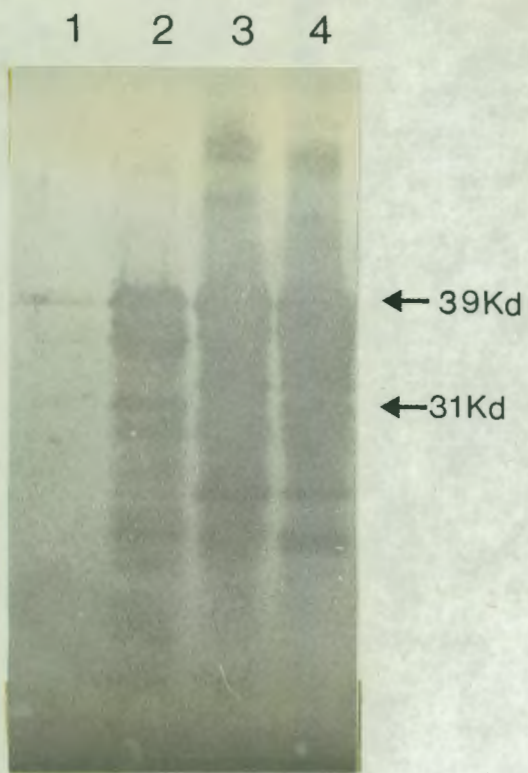
| <u>Ag</u>             | <u>anti-MDMV-A</u> | <u>anti-MDMV-B</u> |
|-----------------------|--------------------|--------------------|
| MDMV-Win              | 0,020              | 2,4                |
| MDMV-ST               | 0,028              | 2,4                |
| Healthy maize control | 0,050              | 0,027              |

This test shows that MDMV-Win and MDMV-ST are related to MDMV-B but not to MDMV-A. The result for MDMV-Win obtained here is verified by that found in Fig. 13.

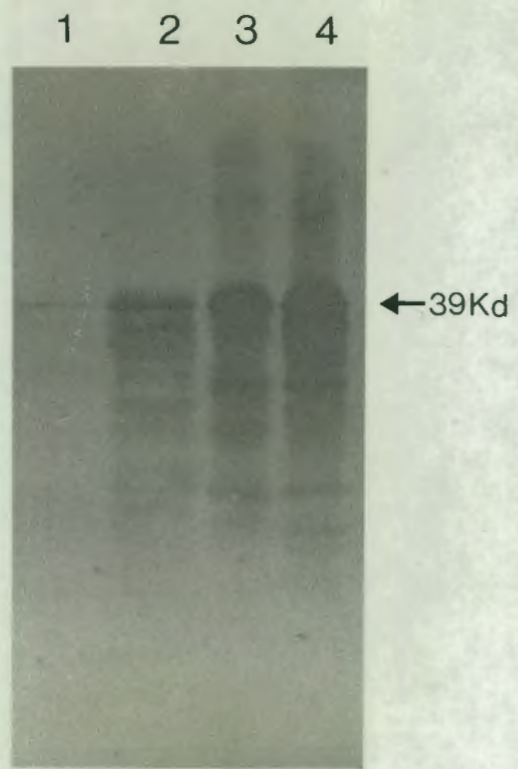
(a) IEB

The immuno-electroblot assay (see section III.G.2) was another test used for investigating the differences or similarities between the MDMV isolates. This test makes use of electrophoretic separation of the viral protein as well as the identification of these proteins by an indirect serological assay. This makes it a very sensitive test for detection of proteins.

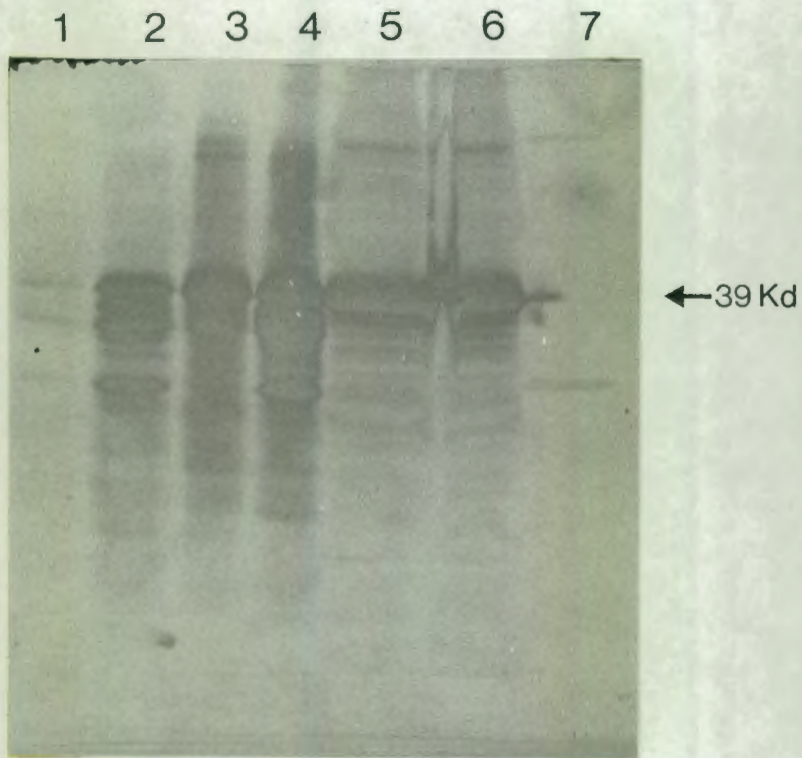
Fig. 14a, b, c, d shows the result obtained in the IEB



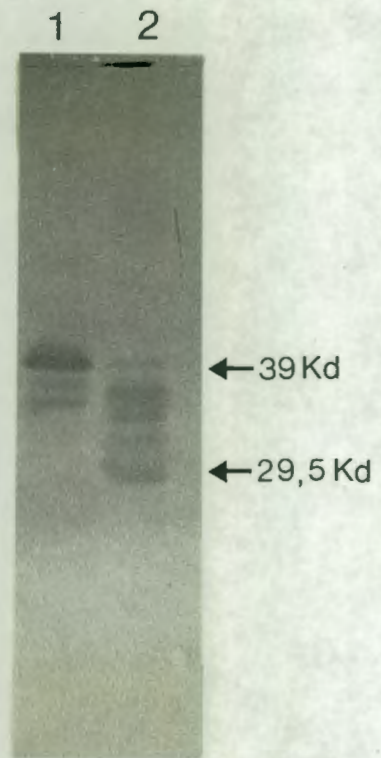
a



b



c



d

Figure 14 :

Immuno-electroblot assays were done with 1/25 dilutions of the following host absorbed antisera.

- a) = anti-MDMV-Kru-1 (early bleeding)
- b) = anti-MDMV-Bar-2
- c) = anti-MDMV-Bar-1
- d) = anti-MDMV-Kru-1 (late bleeding)

The protein samples in each lane are:

Fig. a) lane 1 = SCMV 4975  
2 = MDMV-Win  
3 = MDMV-Kru  
4 = MDMV-Bar

Fig. b) lane 1 = SCMV 4975  
2 = MDMV-Kru  
3 = MDMV-Bar  
4 = MDMV-Win

Fig. c) lane 1 = SCMV 4975  
2 = MDMV-Win  
3 = MDMV-Kru  
4 = MDMV-Bar  
5 = MDMV-Kru infected maize sap  
6 = MDMV-Win infected maize sap  
7 = Molecular weight markers (Pharmacia)

Fig. d) lane 1 = MDMV-Win  
2 = SCMV 4975

assay of MDMV-Win, MDMV-Bar, MDMV-Kru and SCMV 4975. The proteins were reacted with 1/25 dilutions of host absorbed antisera. MDMV-Win, MDMV-Kru and MDMV-Bar isolates were recognised by anti-MDMV-Kru antiserum as shown in Fig. 14a, lanes 2, 3 and 4 respectively. Protein bands at molecular weights of 39Kd to 31Kd were observed. MDMV-Win, MDMV-Kru and MDMV-Bar isolates showed similar polypeptide profiles as shown in Fig. 14a, b and c. SCMV 4975 isolate showed a very slight reaction (Fig. 14a, lane 1). This seems to indicate that SCMV 4975 may be distantly related to the MDMV isolates. Similar results were obtained when SCMV 4975 was reacted with anti-MDMV-Bar-2 and anti-MDMV-Bar-1 antisera as shown in Fig. 14b and c respectively.

A larger number of polypeptides are observed for the three isolates in the IEB assay (Fig. 14c) when compared to the bands obtained on the Coomassie stained SDS-polyacrylamide gel (see Fig. 7, section IV.A.7(b)). The extra bands are lower molecular weight polypeptides in low concentration which are recognized by the antiserum in the IEB assay.

A positive reaction was obtained with SCMV 4975 isolate as shown in Fig. 14d, lane 2. The polypeptide profile of SCMV 4975 is slightly different to that of MDMV-Win.

This test showed that initially when an earlier bleeding

of anti-MDMV-Kru-1 serum was used, there was some difference between the maize isolates and SCMV 4975. One explanation of this difference in reaction in the two tests may be that with time the specificity of the serum would decrease and would therefore recognize closely related antigens. But a more likely explanation is that there may have been a contamination by the seed-transmitted MDMV (see section IV.C). The SCMV 4975 isolate was propagated in this maize and may have been contaminated and therefore been recognized by the anti-serum.

(c) ISEM

An immunosorbent electron microscopy assay (ISEM) was done on samples of maize leaves infected with MDMV-Win and SCMV 4975. The EM grids were coated with 1/1000 dilution of antibody (see section III.F.2 for the assay procedure). Both isolates reacted positively with the anti-MDMV and -SCMV antisera, the filamentous virus particles being trapped with antibody. Figure 15a illustrates a typical reaction, where MDMV-Win particles were coated with anti-MDMV-Win-1 antiserum. Two types of filamentous particles were observed when SCMV 4975 isolate was trapped with anti-MDMV-Kru-2 antiserum. Some particles were positively trapped with negatively stained antibodies surrounding the particles and some virus particles were not trapped positively by antibodies (Fig. 15b). This finding can be explained in two

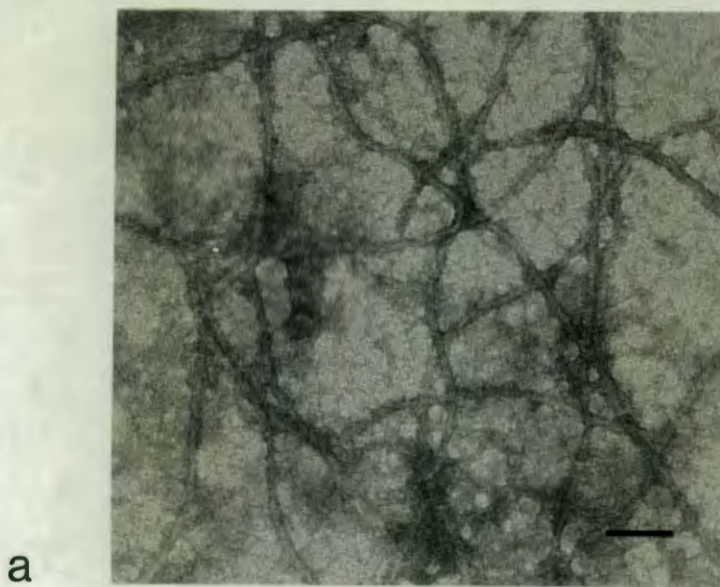


Figure 15 : (a) An ISEM assay showing a typical reaction of MDMV-Win and anti-MDMV-Kru-1 antiserum. The antigen was a partially purified preparation and Ab was diluted 1/1000. The stain was 2% PTA. Magnification is 90 000 x. The bar represents 100nm.

(b) An ISEM assay of SCMV 4975 infected maize leaf sample with anti-MDMV-Kru-2 antiserum (1/1000 dilution) showing two types of filamentous particles. Stain was 2% PTA pH 7,0. The magnification is 60 000 x. The bar represents 200nm.

ways, (a) preparation of the grids, or (b) that actually two species of particles were present. Once the seed transmitted MDMV was discovered, the latter explanation seemed feasible, i.e. that the anti-MDMV-Kru-2 antiserum recognized the contaminating MDMV-ST, whereas the SCMV 4975 is antigenically different as was also shown in IEB assay (see section IV.A.9(b) , Figure 14), when early bleeds of anti-MDMV-Kru and anti-MDMV-Bar were used. (MDMV-ST was shown to be indistinguishable from MDMV-Kru and MDMV-Win (see section IV.C.3)).

Table 15 : The reaction of MDMV-Win and SCMV 4975  
(leaf samples) with MDMV- and SCMV-antisera  
in the ISEM assay

| <u>Antiserum</u> <sup>1</sup> | <u>Antigen</u>  |                         |
|-------------------------------|-----------------|-------------------------|
|                               | <u>MDMV-Win</u> | <u>SCMV 4975</u>        |
| anti-MDMV-Win-1               | + <sup>2</sup>  | +                       |
| anti-MDMV-Win-2               | +               | - <sup>3</sup>          |
| anti-SCMV 4975-1              | +               | +                       |
| anti-MDMV-Kru-1               | +               | +                       |
| anti-MDMV-Kru-2               | +               | +(2 types) <sup>4</sup> |
| anti-MDMV-Bar-1               | +               | +                       |

1. All antisera were diluted at 1/1000 except anti-MDMV-Win-2 and anti-SCMV 4975-1 which were diluted at 1/500
2. + = positive result
3. - = negative result
4. Two types of filaments, coated differently with Ab, were observed.

B. 1. ABRAHAMSKRAAL ISOLATE

Bromus plants found in the ditches on the road west of Bloemfontein leading to Abrahamskraal were infested with Duiraphis noxia aphids and showed typical phytotoxic streaking associated with this aphid. As the aphid-induced phytotoxic symptoms wore off, clear mosaic symptoms remained, indicative of virus infection. Preliminary tests showed that filamentous virus particles could be isolated from infected plants. Prof. M.B. von Wechmar collected these plants and did the initial extraction, transmission and electron microscopy studies.

It was of interest to test whether the mosaic was caused by MDMV infection or by another plant virus. Although sap inoculation to Scheepers wheat gave rise to clear cut mosaic symptoms, inoculation to maize plants resulted in no visible symptoms. The virus isolate was called 'Abrahamskraal' and was propagated on Scheepers wheat, always using inoculum from the original bromus plant.

Maize plants infected with the Abrahamskraal isolate, when examined under the electron microscope, did not show any filamentous particles. Sap inoculated Scheepers wheat examined at the same time, showed the presence of filamentous particles observed earlier (see Fig. 16).

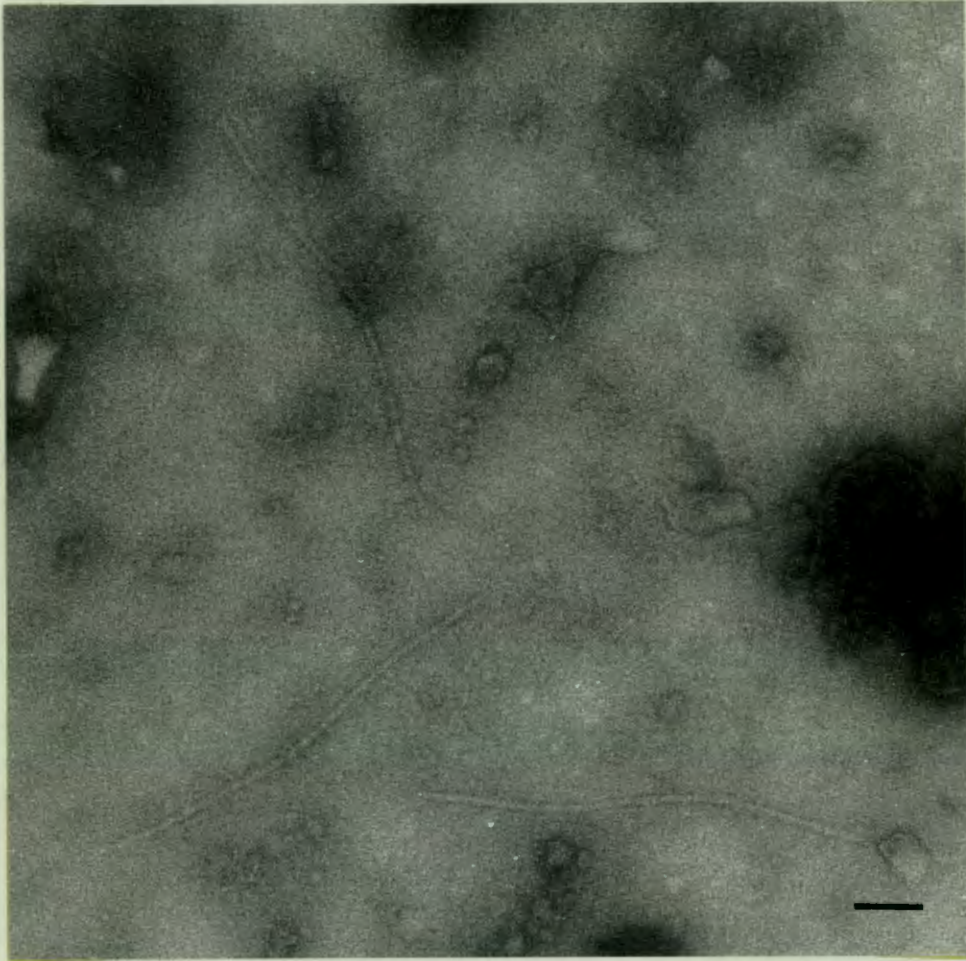


Figure 16 : An electron micrograph of Abrahamskraal virus from Scheepers wheat sap. The stain used was 1% Ammonium molybdate. Magnification 90 000 x. Bar represents 100nm .

MDMV is known to occur in bromus plants (Rosenkranz, 1978). A preliminary serological investigation using the microprecipitin test with anti-MDMV-Win-1 and anti-MDMV-Kru-1 antisera was performed on a partially purified preparation of Abrahamskraal. Titres of 1/64 were obtained with both antisera, whereas homologous titres were 1/1024 and 1/1024 respectively for anti-MDMV-Win-1 and anti-MDMV-Kru-1 antisera. The microprecipitin tests therefore showed that the filamentous particles in bromus plants were distantly related to MDMV-B, or alternatively that the MDMV-antisera contained antibodies recognizing Abrahamskraal virus.

Although the microprecipitin tests were taken as true positives at the time, further tests with ELISA and IEB (see below) did not confirm these reactions, which therefore casts some doubt on this particular result.

The Abrahamskraal isolate was purified from infected Scheepers wheat using the method for MDMV purification (section III.C.2) except where otherwise stated. Antisera to Abrahamskraal was raised. The homologous titres as determined in the microprecipitin test, were 1/512 for both anti-Abrahamskraal-1 and anti-Abrahamskraal-2 IgG's.

The relationship of Abrahamskraal to MDMV was further investigated with the IEB and ELISA methods.

Control samples for the IEB test in Fig. 17 were uninfected Scheepers wheat and concentrated semi-pure extracts of MDMV-ST and maize grown from apical meristem tissue. The Abrahamskraal isolate, used in this test was purified as in section III.C.2 but the PEG step was omitted.

Fig. 17a and b shows the results after IEB assay of the Abrahamskraal isolate using anti-Abrahamskraal-1 and -2 IgG respectively. Both tests show a strong band recognized by the antiserum at 32,5Kd. The wheat control shows no significant reaction. The anti-MDMV-Win-1 and anti-SCMV 4975-1 IgG did not react with Abrahamskraal (see Fig. 17c and d). Anti-MDMV-Kru-1 IgG reacts with Abrahamskraal, as a band at 32,5Kd is observed (see Fig. 17e). This appears to indicate that there is some immunological cross reactivity/relationship of the Abrahamskraal isolate to MDMV-Kru, but not with the MDMV-Win and SCMV 4975 isolates.

Sandwich ELISA tests were done on crude extracts of Abrahamskraal and on purified samples to test for relationship to MDMV-B. The concentrated samples were diluted 4-fold starting with a concentration of 0,1mg/ml. In both cases the Abrahamskraal isolate showed no reaction with anti-MDMV-Win-1, anti-MDMV-Kru-1 or anti-SCMV 4975-1 IgG.

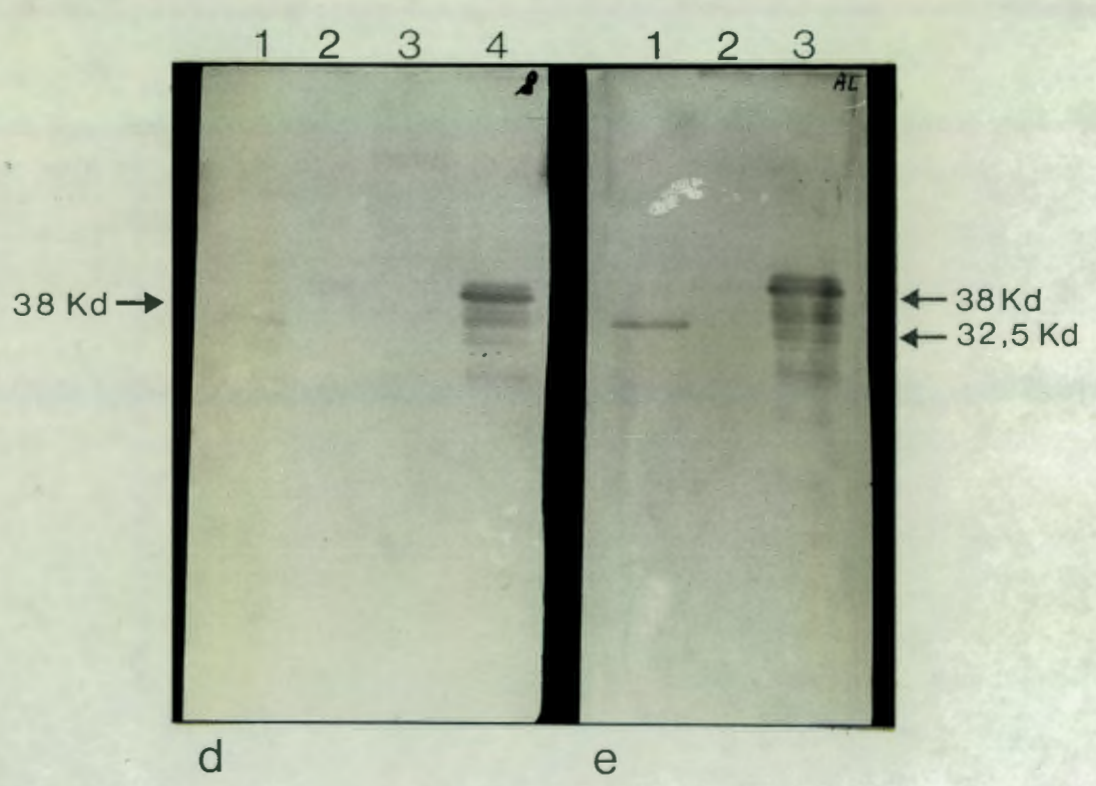
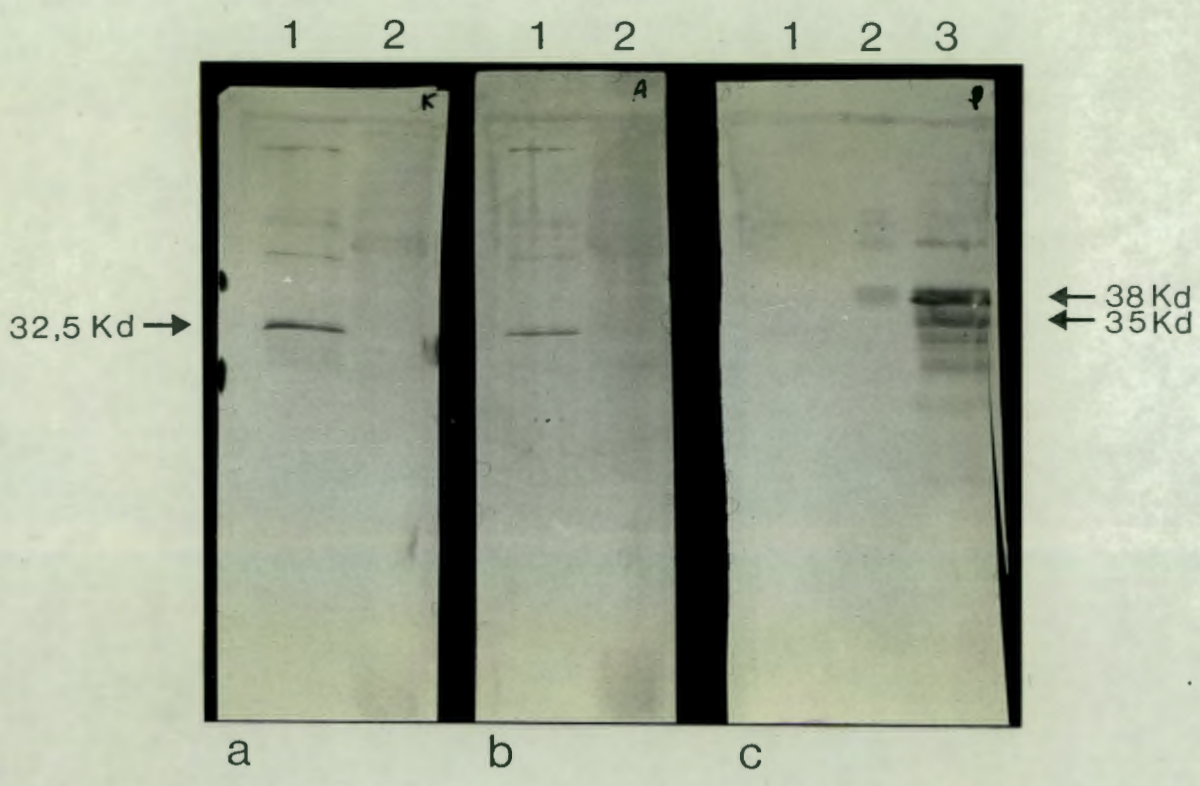


Figure 17 :

The immuno-electroblot assay of Abrahamskraal virus. The blots were probed with the following host absorbed antisera, 1/30 dilution:

- a - anti-Abrahamskraal IgG-1
- b - anti-Abrahamskraal IgG-2
- c - anti-MDMV-Win-1 IgG
- d - anti-SCMV 4975-1 IgG
- e - anti-MDMV-Kru-1 IgG

The antigen samples are in lanes:

- a, lane 1 - Abrahamskraal isolate, partially purified  
(Purification procedure outlined in section III.C.2 but without the PEG-6000 precipitation step)
- lane 2 - Healthy Scheepers wheat sap
- b, lane 1 - Abrahamskraal isolate, partially purified
- lane 2 - Healthy Scheepers wheat sap
- c, lane 1 - Abrahamskraal isolate, partially purified
- lane 2 - Healthy maize, grown from tissue culture, concentrated extract
- lane 3 - MDMV-ST, purified
- d, lane 1 - Abrahamskraal isolate, partially purified
- lane 2 - Healthy maize, grown from tissue culture, concentrated extract
- lane 3 - Healthy Scheepers wheat sap
- lane 4 - MDMV-ST, purified
- e, lane 1 - Abrahamskraal isolate, partially purified
- lane 2 - Healthy maize, grown from tissue culture, concentrated extract
- lane 3 - MDMV-ST, purified

Table 16 : The absorbance values at 405nm obtained in the sandwich ELISA test with Abrahamskraal against anti-MDMV and anti SCMV IgG's

| <u>Antigen</u>   | <u>Antibody</u>        |                        |                         |
|------------------|------------------------|------------------------|-------------------------|
|                  | <u>anti-MDMV-Win-1</u> | <u>anti-MDMV-Kru-1</u> | <u>anti-SCMV 4975-1</u> |
| Abrahamskraal    |                        |                        |                         |
| ex-Scheepers sap | 0,023                  | 0,019                  | 0                       |
| ex-Bromus sap    | 0,005                  | 0,022                  | 0                       |
| concentrated     | 0,113                  | 0,027                  | 0,082                   |
| MDMV-ST          |                        |                        |                         |
| ex-maize sap     | 2,35                   | 1,521                  | 1,118                   |
| concentrated     | 2,5                    | 2,5                    | 2,7                     |
| Maize control    |                        |                        |                         |
| sap              | 0,083                  | 0,019                  | 0,253                   |
| concentrated     | 0,235                  | 0,082                  | 0,064                   |

The above negative results do not agree with results obtained with the microprecipitin and IEB tests.\* Reciprocal tests were not done, as at this time anti-Abrahamskraal was not yet available. In fact, these tests emphasized the need for antiserum to this unidentified virus.

Further IEB tests were done on the Abrahamskraal isolate using anti-Barley yellow mosaic virus (BaYMV) antiserum

\* This is probably due to the pronounced homologous antigen specificity of the sandwich ELISA.

raised against extracts of dessicated infected leaves obtained from Dr. S. Hill (Cambridge) and anti-wheat streak mosaic virus (WSMV) antiserum obtained from M.K. Brakke. Antisera and IgG used were host absorbed. Fig. 18a shows that anti-MDMV-Win-1 IgG reacted very weakly with a protein of Abrahamskraal at the 37Kd position. Fig. 18b shows the homologous reaction of Abrahamskraal with anti-Abrahamskraal-2 IgG. The major bands are at the 32,5 and 26,5Kd positions. Fig. 18c shows no reaction of the Abrahamskraal isolate with anti-BaYMV antiserum. No relationships could thus be found between Abrahamskraal and BaYMV. The antiserum to BaYMV was of a poor quality and further work is necessary before a valid result can be obtained. When Abrahamskraal was reacted with anti-WSMV-antiserum, a weak positive reaction was obtained (Fig. 18d). Two bands, one at the 38,5Kd and 26,8Kd positions respectively, were observed. Proteins in the BaYMV isolate were recognized by anti-WSMV antiserum but their identity was not resolved.

More complete investigations are necessary to resolve the identity of Abrahamskraal virus and its possible relationship to other filamentous viruses such as WSMV and BaYMV.

The MDMV-Win and MDMV-ST isolates showed negative results when tested with anti-Abrahamskraal-2 IgG,

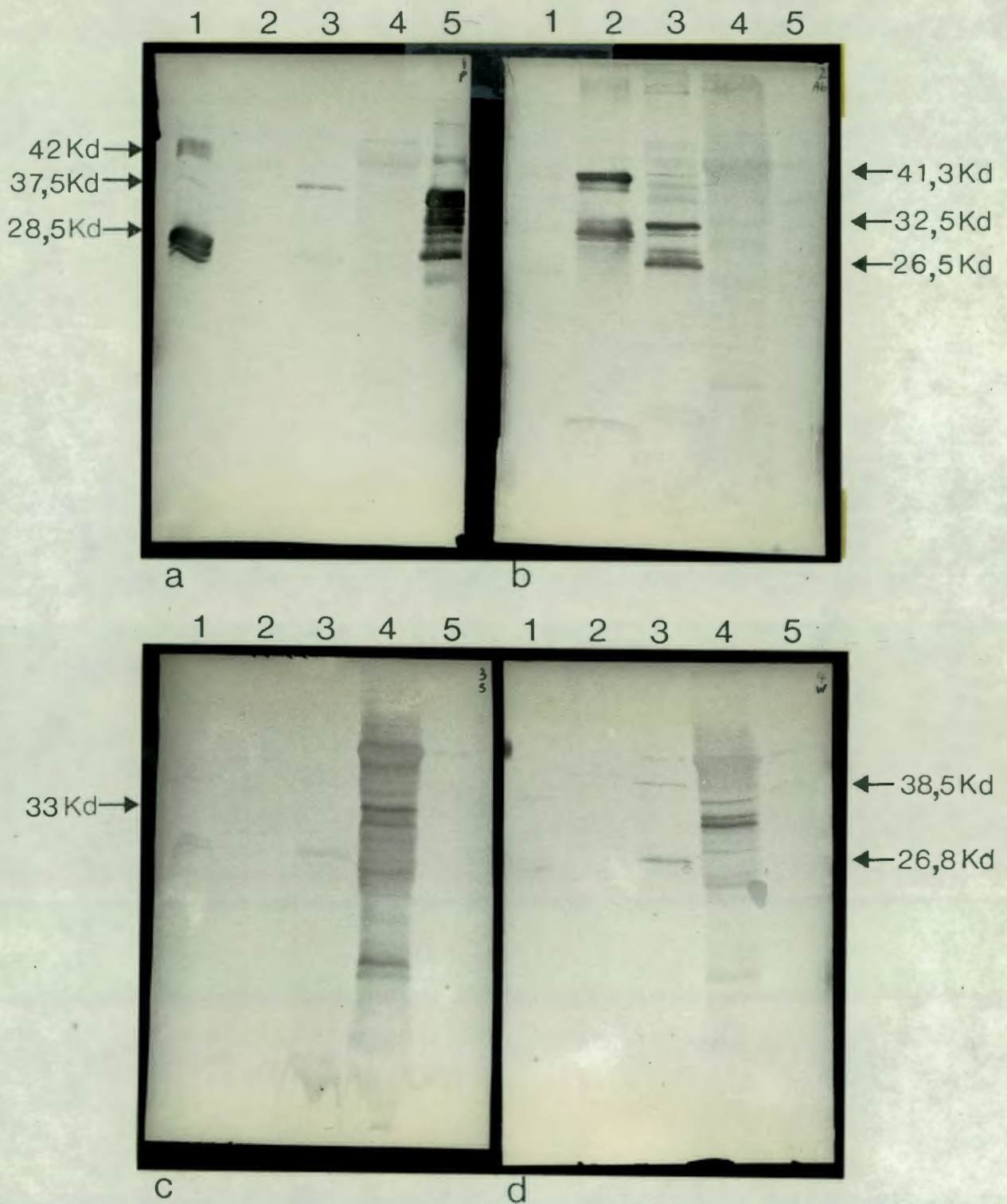


Figure 18 :

The immuno electroblob test of the Abrahamskraal isolate using the following host absorbed antisera:

- a = anti-MDMV-Win-1 IgG
- b = anti-Abrahamskraal-2 IgG
- c = anti-BaYMV antiserum
- d = anti-WSMV antiserum

Antigens in the lanes are:

- 1 = MDMV-Win
- 2 = Barley isolate-1
- 3 = Abrahamskraal
- 4 = Crude sap of BaYMV infected barley
- 5 = MDMV-ST

anti-BaYMV and anti-WSMV antisera (Fig. 18b, c and d), in contrast to anti-MDMV-Kru-1 which reacted positively with Abrahamskraal (Fig. 17e). (For description of MDMV-ST, see Seed-transmission, section IV.C).

(See also section IV.B.3a for description of reactions of Barley isolate-1 in Fig. 18).

The Abrahamskraal isolate was tested in the indirect ELISA test using the anti-WSMV antiserum. Antigen was diluted 4-fold with a starting concentration of the Abrahamskraal isolate at 0,1mg/ml. The anti-WSMV antiserum was used at 1/100 dilution and the alkaline phosphatase goat-anti-rabbit IgG conjugate at 1/1000 dilution. The control antiserum used for this test was raised against Tobacco mosaic virus (TMV) and used at 1/100 dilution.

Table 17 shows the result of this test, with the Abrahamskraal isolate showing a strong positive reaction with anti-WSMV antiserum.

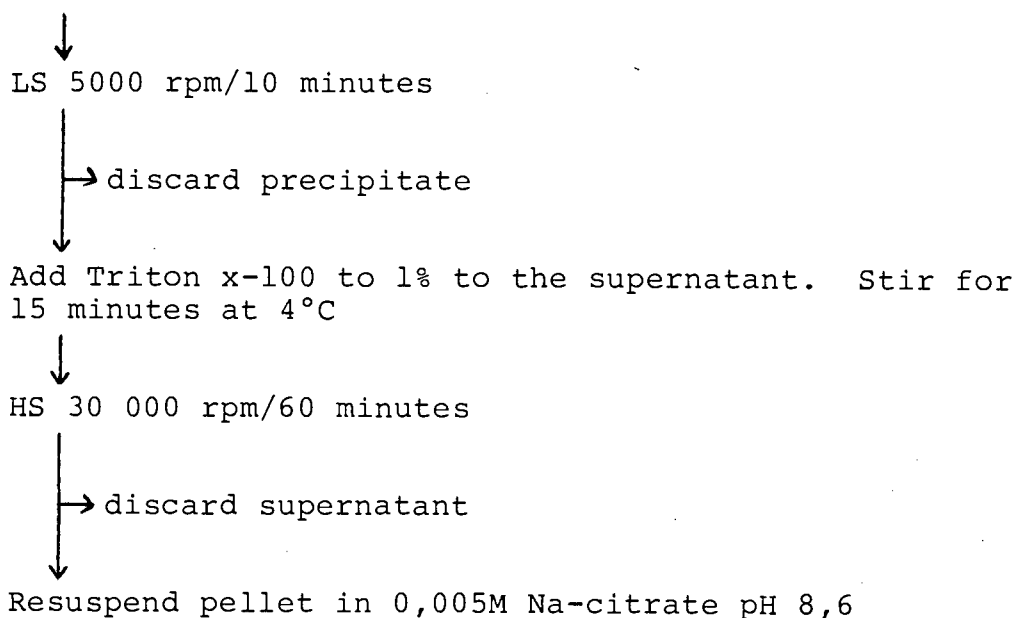
Table 17 : The absorbance values at 405nm of  
Abrahamskraal reacted with anti-WSMV-anti-  
serum in the indirect ELISA

| <u>Antigen</u>              | <u>Absorbance at 405nm</u> |                           |
|-----------------------------|----------------------------|---------------------------|
|                             | <u>anti-WSMV-antiserum</u> | <u>anti-TMV-antiserum</u> |
| Abrahamskraal<br>(0,1mg/ml) | 1,198                      | 0,150                     |
| Scheepers wheat<br>sap      | 0,640                      | 0,115                     |

There was a high background absorbance value for the Scheepers wheat control. There was no dilution effect, with the absorbance value remaining at 0,5 with an increase in dilution of sap. The high background reading was obtained because the anti-WSMV antiserum was not host absorbed and therefore contained antibodies to the host protein. Anti-TMV antiserum control reacted negatively with both the Abrahamskraal isolate and the wheat control.

For further ELISA tests the Abrahamskraal isolate was purified by the method used for MDMV purification, resuspending the virus in 0,05M Borate pH 8,0 and 0,1M KPO<sub>4</sub> pH 7,0. The isolate was also purified using Na-citrate pH 8,6. This purification procedure was adapted from van Regenmortel et al (1962) and is described overleaf.

Infected leaves were ground in 0,05M Na-citrate pH 8,6  
3g leaf weight : 4ml buffer



The above preparations of Abrahamskraal were tested in the sandwich ELISA test against anti-MDMV antisera.

The presence of spherical particles in preparations of the Abrahamskraal isolate, as seen in electron micrographs, was thought to be a possible contamination by cucumber mosaic virus (CMV). This was tested by purifying the Abrahamskraal isolate from wheat cv. Scheepers using the purification procedure for CMV, Mossop et al, (1976). This product and the Abrahamskraal isolate purified by using the method for MDMV purification, was tested against anti-CMV antisera (kindly provided by Pumezo Lupuwana) in the sandwich ELISA test. The results are given in Table 18.

Table 18 : The absorbance at 405nm obtained when the Abrahamskraal isolate was tested against MDMV- and CMV-antisera in the sandwich ELISA

| Antigen                                                                   | Absorbance at 405nm |                     |                     |                |                |  |
|---------------------------------------------------------------------------|---------------------|---------------------|---------------------|----------------|----------------|--|
|                                                                           | anti-MDMV<br>-Win-1 | anti-MDMV<br>-Kru-1 | anti-SCMV<br>4975-1 | anti-<br>CMV-1 | anti-<br>CMV-2 |  |
| <u>Test 1</u>                                                             |                     |                     |                     |                |                |  |
| Abrahamskraal isolate purified by<br>'MDMV method' in 0,05M Borate pH 8,0 | 0,006               | 0,011               | 0,014               | -1             | -              |  |
| Abrahamskraal in 0,1M PO <sub>4</sub> pH 7,0                              | 0,007               | 0,003               | 0,002               | -              | -              |  |
| Abrahamskraal in 0,005M Na-citrate pH 8,6                                 | 0,003               | 0,005               | 0,018               | -              | -              |  |
| MDMV-Win                                                                  | 2,4                 | 1,938               | 2,7                 | /              | /              |  |
| Concentrated healthy maize                                                | 0,417               | 0,193               | 0,066               | /              | /              |  |
| CMV infected sap                                                          | /                   | /                   | /                   | +              | +              |  |
| Healthy tobacco sap                                                       | /                   | /                   | /                   | -              | -              |  |
| <u>Test 2</u>                                                             |                     |                     |                     |                |                |  |
| Abrahamskraal isolate purified by<br>'MDMV method' 0,1mg/ml               | 0,004               | /                   | /                   | 0,000          | /              |  |
| MDMV-Win 0,12mg/ml                                                        | 2,4                 | /                   | /                   | 0,002          | -              |  |
| Concentrated preparation of healthy<br>maize grown from tissue culture    | 0,000               | /                   | /                   | 0,000          | /              |  |
| CMV infected sap                                                          | 0,12                | /                   | /                   | 2,9            | /              |  |
| Healthy tobacco sap                                                       | 0,006               | /                   | /                   | 0,007          | /              |  |

Test 3

Abrahamskraal isolate purified by  
'CMV method' 0,15mg/ml

|                     |       |   |   |       |   |
|---------------------|-------|---|---|-------|---|
| CMV infected sap    | 0,049 | / | / | 0,019 | / |
| Healthy tobacco sap | /     | / | / | 0,508 | / |
| MDMV-Win 0,15mg/ml  | 2,9   | / | / | 0,033 | / |
| Healthy maize sap   | 0,133 | / | / | /     | / |

---

l = results determined visually    - = negative result    + = positive result    / = not tested

The negative results reflected in Table 18 show that either CMV is not present or that the abovementioned purification methods were not suitable for isolating a possible CMV-isolate from Abrahamskraal.

The Abrahamskraal isolate did not react with anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-SCMV 4975 in the ELISA tests. This is in contrast with a positive reaction observed in the IEB assay where Abrahamskraal reacted positively with anti-MDMV-Kru-1 (see Fig. 17e). Final conclusions can only be made once reciprocal tests are done with the relevant antigens and antisera.

An ISEM examination of Abrahamskraal with homologous antisera and anti-MDMV antisera was not satisfactory, mainly due to the fact that very few filamentous particles could be found in leaf squash preparations and was therefore not pursued.

## 2. JOHNSONGRASS ISOLATES

### (a) Johnsongrass isolate-1

Johnsongrass (Sorghum halepense (L) Pers) is frequently found along the road sides in the maize growing areas in South Africa. It is known to be an important overwintering host for MDMV-A in the United States, acting as a virus source for the next seasonal maize crop (Gordon et al, 1981). It was of interest to investigate

whether MDMV is found in Johnsongrass in South Africa.

A sample of a naturally infected Johnsongrass plant showing mosaic symptoms was collected near Brits in the Transvaal by Prof. M.B. von Wechmar during a field trip in 1984.

The virus from this sample was mechanically transmissible to maize plants, producing typical mosaic symptoms.

The virus was strongly infectious showing mosaic symptoms on 17/19 plants 13 days post-inoculation. No symptoms were produced when sugarcane plants were inoculated with this isolate. (Virus-free sugarcane plants produced by tissue culture (part of another project) were used. See section III.B.4(c)2).

Leaf dip preparations of maize plants infected with Johnsongrass isolate-1 were examined under the electron microscope. Filamentous particles were observed (Fig. 19a). An ISEM assay on this sample using anti-MDMV-Win-1 IgG, showed negative results, i.e. the particles were not trapped by the anti-MDMV-strain B antibodies (see Fig. 19b). This result indicates that Johnsongrass isolate-1 was different to MDMV-Win (or MDMV-strain B) and could possibly be an isolate of MDMV-A. The obvious next step was to test for MDMV-A but no anti-MDMV-A serum was available at this time. The serum bleeds were too young for use here.

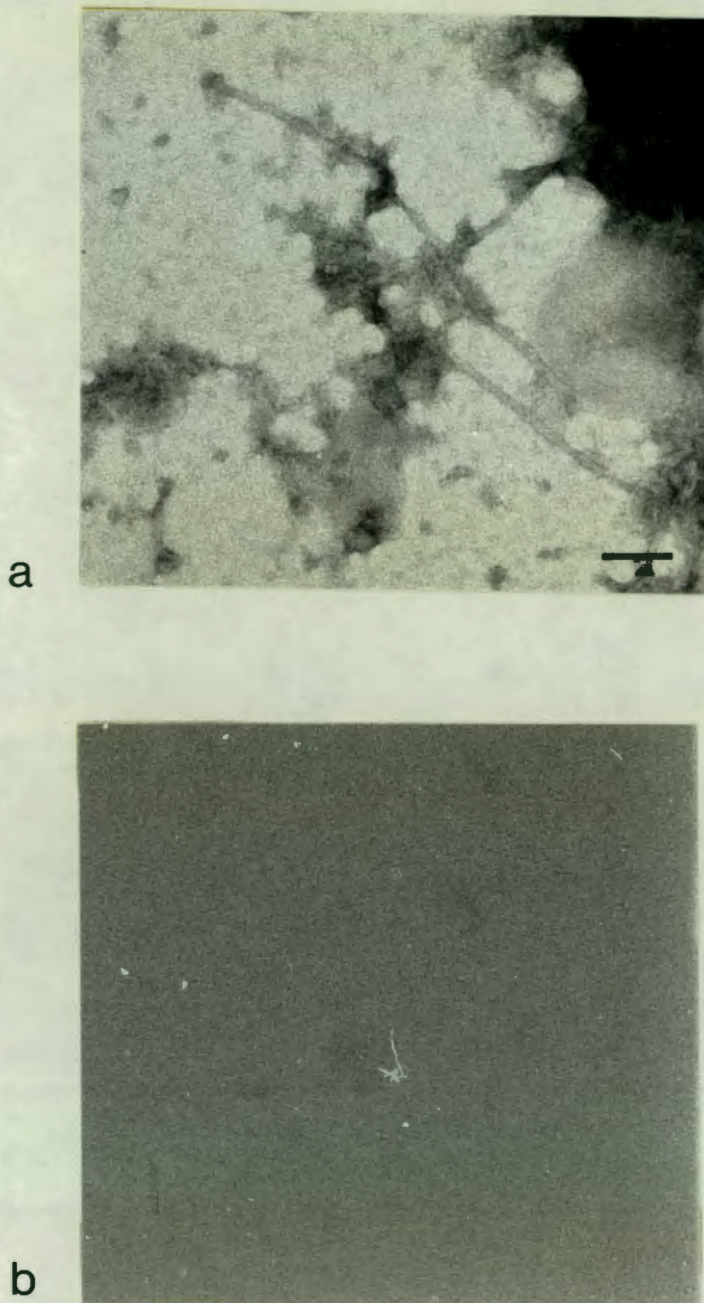


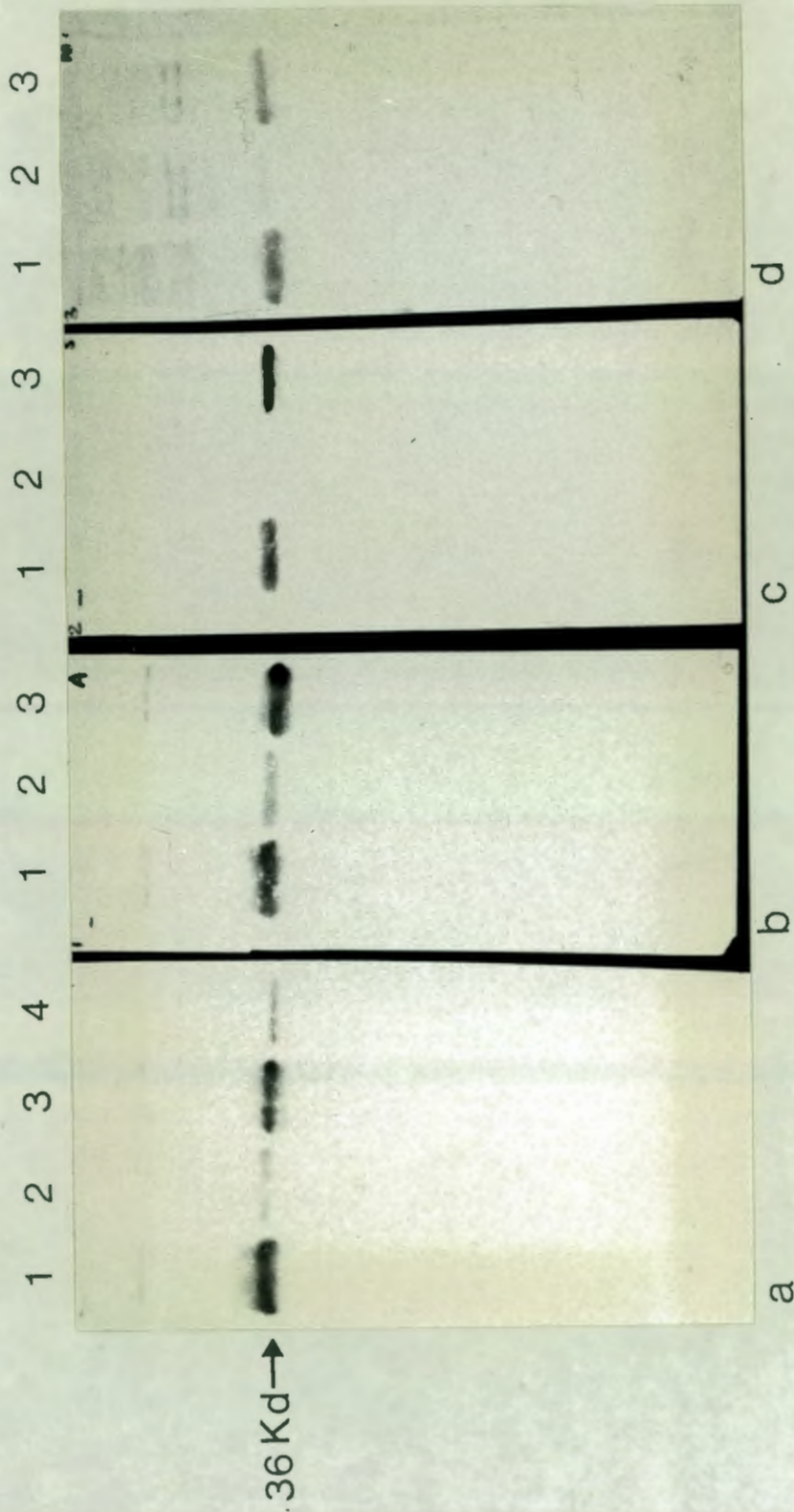
Figure 19 :

a. Electron micrograph of a leaf squash preparation of Johnsongrass-isolate-1 infecting maize (MDMV-A). The stain was 2% uranyl acetate pH 6. Magnification was 90 000X. (The bar represents 100nm)

b. The above sample tested with heterologous anti-MDMV-Win-1 IgG (=anti-MDMV-B) (1/1000 dilution) in an ISEM assay.

Johnsongrass isolate-1 was further tested against MDMV-strain-B antisera at 1/80 dilution in the IEB assay. A crude preparation of the isolate was used. Johnsongrass leaves showing mosaic symptoms were ground in 0,1M  $KPO_4$  pH 7,0 buffer at 1 : 2 w/v ratio. This was filtered through cheesecloth and debris was removed by LS centrifugation. The sample was dissociated 1 : 1 in dissociation buffer. Johnsongrass isolate -1 reacted positively with the anti-MDMV-Win-1 and anti-MDMV-Kru-1 IgG showing a single band at 36Kd position as shown in Fig. 20a and b. This band was of very low intensity compared with the band obtained in the homologous reaction with MDMV-Kru and anti-MDMV-Kru-1 (Fig. 20b). A similar low intensity reaction was obtained with anti-MDMV-Win-1 IgG (Fig. 20a). Johnsongrass isolate-1 did not react with anti-SCMV 4975-1 and anti-MDMV-Bar as shown in Fig. 20c and d. In the reaction with anti-MDMV-ST antiserum, Johnsongrass isolate-1 reacts positively but with a lesser intensity than the MDMV-ST/ anti-MDMV-ST IgG reaction as shown in Fig. 21. Johnsongrass isolate-1 again reacts as a single component but at the 35Kd position which is at a slightly lower MW position than the major band of MDMV-ST which is at the 36Kd position. (See Seed transmission, section IV.C, for a description of MDMV-ST).

The above results indicate that Johnsongrass isolate-1 showed some relationship, but very weak, to MDMV-Win,



**Figure 20 :**

IEB assay of Johnsongrass isolate-1 and controls. The immuno-electroblots were assayed with the following IgG at 1/80 dilutions:

- a - anti-MDMV-Win-1
- b - anti-MDMV-K-1
- c - anti-SCMV 4975-1
- d - anti-MDMV-Bar-1

Lanes 1 - 3 of a - d show the following samples:

- 1 - MDMV-ST (crude preparation from sap)
- 2 - Johnsongrass isolate-1 (crude preparation from sap)
- 3 - MDMV-K (crude preparation from sap)

Lane 4, a - MDMV-ST (concentrated preparation after differential ultra-centrifugation)

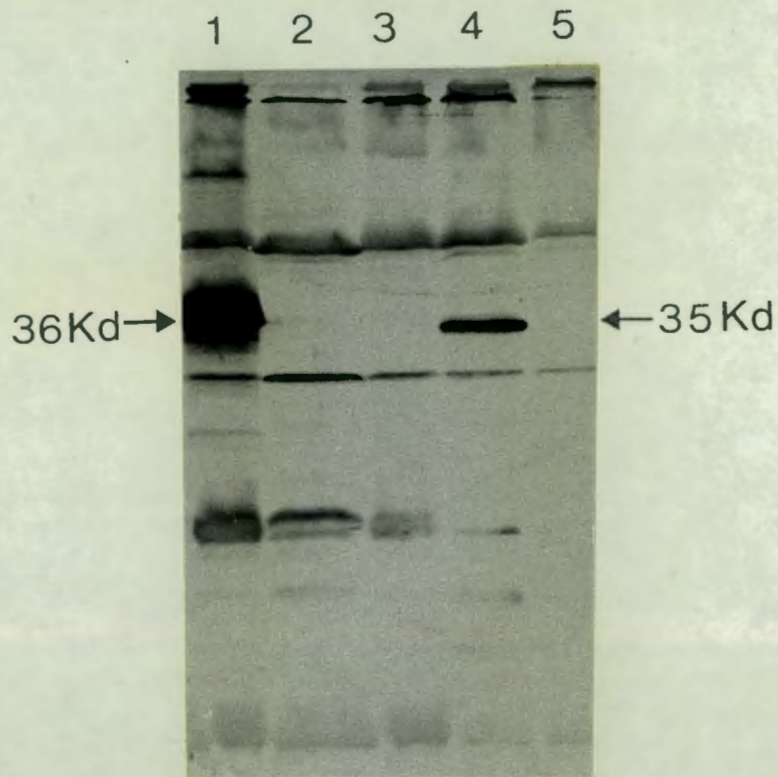


Figure 21 :

IEB of Johnsongrass isolate-1 and controls with anti-MDMV-ST IgG.

- Lane 1 - MDMV-ST (crude preparation from sap)
- Lane 2 - Healthy sugarcane (crude preparation from sap)
- Lane 3 - Healthy maize (crude preparation from sap)
- Lane 4 - Johnsongrass isolate-1 (crude preparation from sap)
- Lane 5 - Healthy Johnsongrass (crude preparation from sap)

MDMV-Kru and MDMV-ST which are all strongly related to MDMV-B. It is interesting to note that the MDMV-B specific antisera, anti-MDMV-Win-1, -Kru and -ST recognized a strain of MDMV different to strain B in the IEB assay. MDMV-B and the other Johnsongrass infecting strains, A, C, D, E, F were shown to be serologically related by Louie and Knoke (1975) and Snazelle et al (1971) using the microprecipitin test. Regrettably these workers did not follow this up with more sensitive serological techniques, e.g. ELISA. Langenberg (1974) also showed that MDMV-A and -B are serologically related but not identical, using the leaf-dip serology technique. Furthermore, in the IEB assay, related strains of a virus can be detected by antiserum to one of the strains. This was shown by Rybicki and von Wechmar (1982) for strains of TMV.

In Fig. 20a, b, c and d MDMV-Kru (lane 3) reacts as strongly with all the antisera, as MDMV-ST (lane 1), which shows that these two isolates are closely related.

The homologous reaction with MDMV-ST (a crude preparation from maize sap) with anti-MDMV-ST IgG is shown in Fig. 21, lane 1. The controls, uninfected maize sap and uninfected Johnsongrass sap, in lanes 3 and 5 respectively, do not show the reaction at the 36Kd position, showing that no virus could be detected in unconcentrated saps. The bands at positions 51Kd,

29Kd, 18Kd and 16Kd are host proteins reacting with the antiserum as these are present in the infected and uninfected samples. This indicates that the IgG contains antibodies to host protein, which are detected in this sensitive assay indicating that host absorption was not complete.

(b) Johnsongrass isolate-2

A second Johnsongrass isolate from the U.S. (obtained as lyophilized leaf tissue) was tested against the local MDMV antisera in the IEB assay. This test was done only on lyophilized tissue as the isolate was not propagated.

This isolate was prepared by grinding leaf tissue in 10x volume of 0,1M  $KPO_4$  pH 7,0 buffer and removing plant debris by LS centrifugation. The supernatant was concentrated by centrifuging at HS and the pellets obtained were resuspended in  $KPO_4$  buffer. The volume of buffer used was in a ratio equal to the original weight of sample. Purified MDMV-ST was used as a positive control in the IEB assay.

Johnsongrass isolate-2 was recognized by anti-MDMV-Win-1 and anti-MDMV-Kru-1 IgG as a single band with an approximate MW of 37Kd. The reaction obtained is shown in Fig. 22b for anti-MDMV-Win-1 IgG. Anti-MDMV-ST IgG also reacted positively with this isolate. The virus

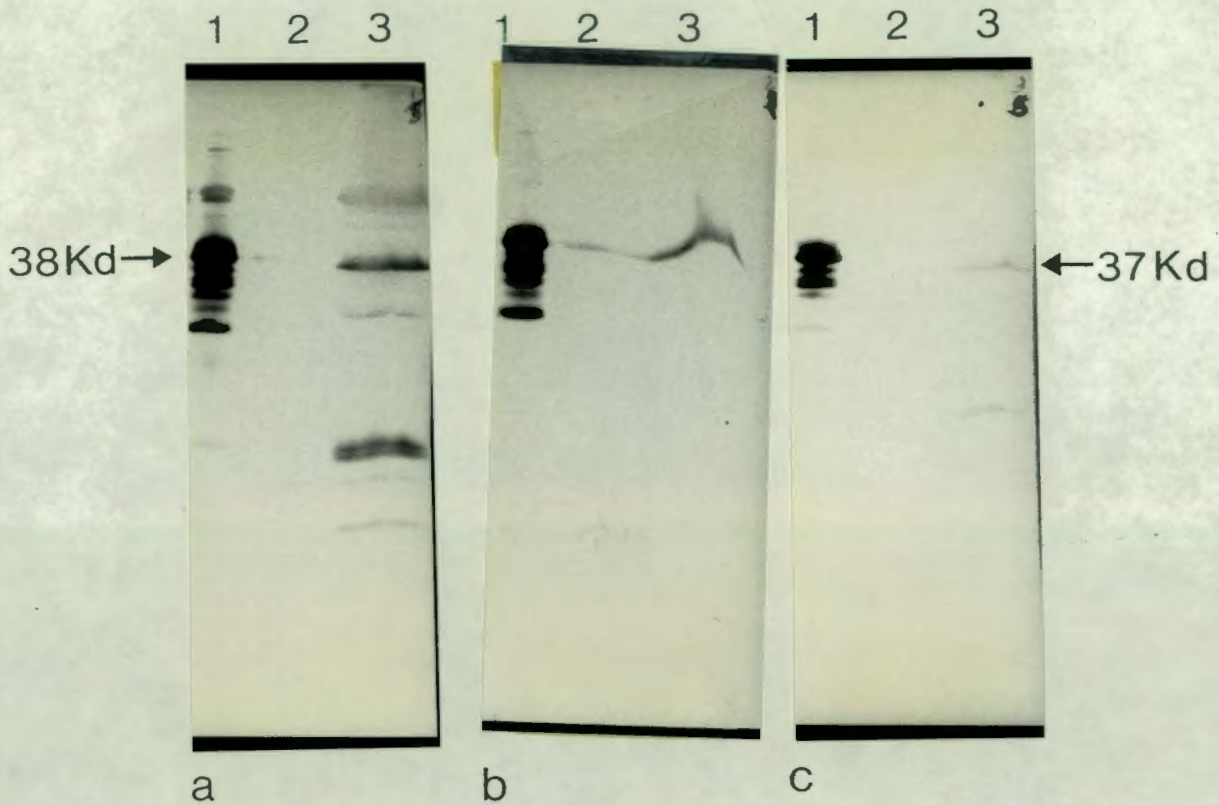


Figure 22 :

IEB of Johnsongrass isolate-2 and barley isolate-2 against various MDMV antisera.

a - anti-MDMV-ST

b - anti-MDMV-Win-1

c - anti-SCMV 4975-1

The samples were:

Lane 1 - MDMV-ST

Lane 2 - Barley isolate-2

Lane 3 - Johnsongrass isolate-2

protein band is at the 37Kd position as shown in Fig. 22a. The other bands in this sample, at 58Kd, 33Kd, 21,5Kd, 20,5Kd and 16,2Kd positions are host protein reactions. This particular preparation of IgG detected host protein in IEB assay, see Fig. 21. The Johnsongrass isolate-2 was very weakly or non-specifically recognized by anti-SCMV 4975 IgG as shown in Fig. 22c.

These results showed that Johnsongrass isolate-2 reacted more strongly with the anti-MDMV-Win, -Kru and -ST antisera than with anti-SCMV 4975. (See section on Barley isolates, IV.B.3, for an explanation of reactions in Fig. 22 of Barley isolate-2 with MDMV antisera).

Sandwich ELISA tests were done on this preparation of Johnsongrass isolate-2 with anti-MDMV-Win-1 and anti-MDMV-ST IgG. Positive and negative controls were dessicated maize tissue infected with MDMV-Win and fresh uninfected maize leaves. These were prepared in the same way as the infected Johnsongrass isolate-2 sample as discussed earlier. This isolate showed negative results with both antisera. The absorbance readings at 405nm for the first 5-fold dilution, are shown in Table 19.

Table 19 : The absorbance readings at 405nm obtained  
for Johnsongrass isolate-2 and controls  
tested in the sandwich ELISA with MDMV  
antibodies

|                           | <u>anti-MDMV-Win-1</u> | <u>anti-MDMV-ST</u> |
|---------------------------|------------------------|---------------------|
| Johnsongrass<br>isolate-2 | 0,830                  | 0,803               |
| Healthy<br>maize          | 0,607                  | 0,791               |
| MDMV-Win                  | 2,75                   | 2,75                |

### 3. BARLEY ISOLATES

#### (a) Barley-Isolate-1

Barley-isolate-1 was given to me from the Gramineae virus collection maintained in the Department. The virus was routinely propagated by aphid transmission and preliminary investigations had shown that it contained a mixture of spherical and filamentous particles. It was known that one of the spherical viruses was BMV. The filamentous particles were as yet unidentified. The isolate was included in this project to ascertain whether the filamentous aphid transmitted virus was related to MDMV. Barley Hordeum vulgare L. is not a known host of MDMV but Gates (1983) found that symptomless infections could occur in barley.

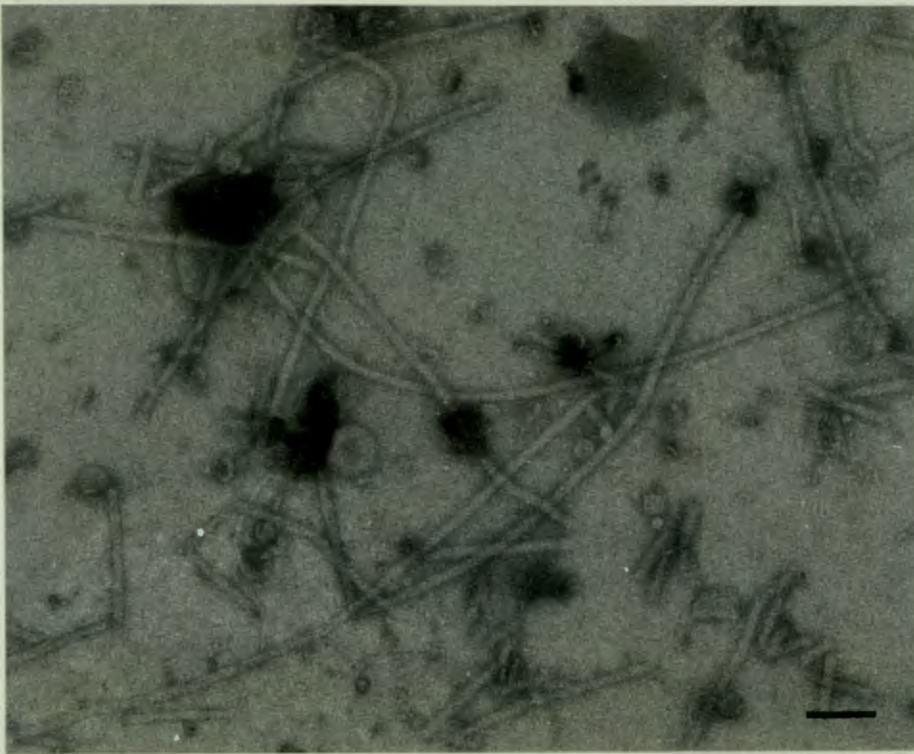
The barley isolate-1 was tested in the microprecipitin tests with MDMV-Win-1 and MDMV-Kru-1 antisera. A sample of barley isolate-1 was clarified in 10% chloroform and resuspended in 0,1M  $KPO_4$  pH 7,0. When tested with anti-MDMV-Win-1 antiserum a titre of 1/64 was obtained. The barley isolate-1 was also absorbed with anti-maize Fraction I serum at 5:1 dilution to remove host antigens in the preparation that could cross react with anti-maize host antibodies. After LS centrifugation the Barley isolate-1 was tested in a

microprecipitin test. A titre of 1/16 was obtained with anti-MDMV-Win-1 host absorbed IgG.

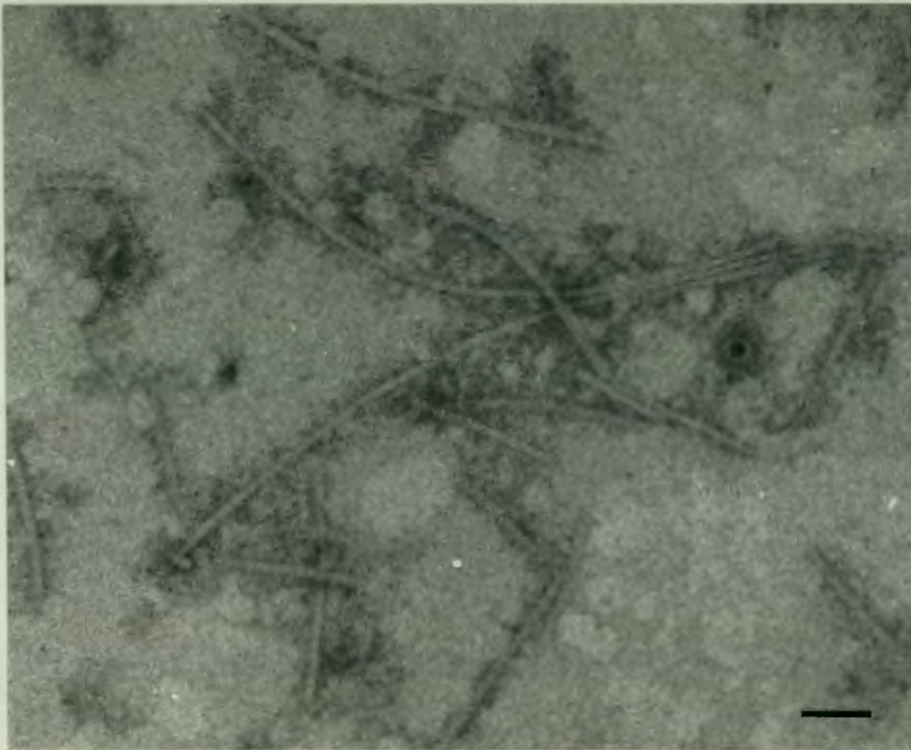
Ouchterlony double diffusion tests with sap from plants infected with barley isolate-1 and anti-MDMV-Win-1, anti-SCMV 4975 and anti-BMV sera showed a strong reaction with the anti-BMV serum indicating that the isolate also contained BMV.

Reactions of the filaments with MDMV-Win-1 were also visible as precipitin bands close on the antigen well periphery after three days. Results not shown.

The Barley isolate-1 sample after sucrose density gradient purification, examined in the electron microscope, revealed the presence of filamentous particles as well as spherical and rod shaped particles, (see Fig. 23a). An ISEM assay with 1/1000 dilution of anti-MDMV-Win-1 IgG showed a positive reaction where filamentous particles were trapped with antibody (Fig. 23b). The same test with anti-Abrahamskraal-2 IgG showed negative results. The Barley isolate-1 was also tested in the IEB assay with anti-MDMV-Win-1, anti-BaYMV, anti-WSMV and anti-Abrahamskraal-2 antibodies. It did not show any reaction with the first three antisera. Protein bands at the 41,3Kd



a



b

Figure 23 : (a) Electron micrograph of Barley isolate-1. The negative stain was 2% uranyl acetate. The bar represents 100nm.  
(b) ISEM of Barley isolate-1 and 1/1000 dilution of anti-MDMV-Win-1 IgG. The bar represents 100nm.

and 31Kd positions were detected with the anti-Abrahamskraal-2 IgG (see Fig. 16, section IV B 1). A crude preparation of Barley isolate-1 was tested with anti-SCMV 4975-1 antibody in the sandwich ELISA test. A weak reaction was obtained, as shown in Table 20.

Table 20 : The results obtained in the sandwich ELISA when Barley isolate-1 was tested with anti-SCMV 4975-1 IgG.

| <u>Antigen</u>                       | <u>Absorbance at 405nm<sup>1</sup></u> |
|--------------------------------------|----------------------------------------|
| Barley isolate-1 (crude preparation) | 0,396                                  |
| Uninoculated maize sap               | 0,226                                  |
| MDMV-Win infected maize sap          | 1,484                                  |

1. Average of 2 readings.

The above results were not consistent. The microprecipitin and ISEM assay showed that the Barley isolate-1 cross-reacted with antibodies in anti-MDMV-Win-1, but according to the IEB assay, the Barley isolate-1 was not related to MDMV-Win but was recognised by Abrahamskraal antibodies. Therefore further tests with homologous and heterologous

reactions with Abrahamskraal, MDMV and Barley-isolate-1 and their respective antisera are required to establish possible relationships, or to identify possible mixtures of viruses that occur in graminaceous hosts.

(b) Barley Isolate-2

Another barley virus isolate was obtained from the Departmental collection maintained by Professor von Wechmar. Barley cv. Clipper grown from a particular seed batch and used for barley yellow dwarf virus (BYDV) multiplication (by sequential aphid transmission), contained filamentous particles when processed for BYDV. These particles were isolated as a faster sedimenting fraction after rate zonal centrifugation on a 10-50% sucrose gradient. The filamentous particles (Barley isolate-2) were further investigated here. It was propagated on barley cv. Clipper (aphid transmission) and was purified using the MDMV purification method (see section III.C.2). Electron micrographs of the purified Barley isolate-2 showed filamentous virus-like particles and isometric particles.

This isolate was tested in microprecipitin tests with anti-MDMV-Win-1 and anti-MDMV-Kru-1 antibodies. To ensure that no host reaction occurred, Barley isolate-2 was absorbed with anti-maize Fraction 1 antiserum.

After precipitating the reacted antibodies by a LS centrifugation step, the Barley isolate-2 was reacted with two-fold dilutions of antiserum. Negative reactions for both antibodies were obtained.

Barley isolate-2 was tested with anti-MDMV-ST, anti-MDMV-Win-1, anti-SCMV 4975 antibodies in the IEB test, as shown in Fig. 22 a,b and c respectively, (section IV.B.26). No reaction was observed with anti-MDMV-ST nor anti-SCMV 4975 IgG's (Fig. 22 lanes 2, a and c respectively). The weak protein reaction present in Fig. 22b, lane 2, with MDMV-Win-1, is ambiguous. This band is clearly a continuation of the protein band in lane 3 and most likely arose through contamination of the barley sample from lane 3.

It can be concluded from the above results that no relationship exists between MDMV and the Barley isolate-2. The identity of this isolate thus remains unresolved and would need further investigation at a later time. As it is not part of the MDMV-project, it was not pursued further.

## C. SEED TRANSMISSION

### 1. INTRODUCTION

During the course of this investigation, it had been a routine practice to select the most vigorously growing maize seeds for use as inoculation host for the routine propagation of MDMV. After germination in moist vermiculite for two days, the fast germinating seedlings were selected for planting out. Occasionally a plant would show a typical mosaic symptom, although it was uninoculated. When slow germinating maize was planted, a large proportion of these plants showed abnormal growth, such as stunting and dead growing points. See section IV.A, Table 6 and Fig. 2. These observations and the fact that sometimes extracts of uninoculated maize tested with host absorbed MDMV antiserum gave high background readings, gave rise to the hypothesis that a seed transmitted virus could be present.

#### Healthy maize tested with MDMV antiserum

To test for the presence of MDMV in apparently healthy maize, the following procedure was followed.

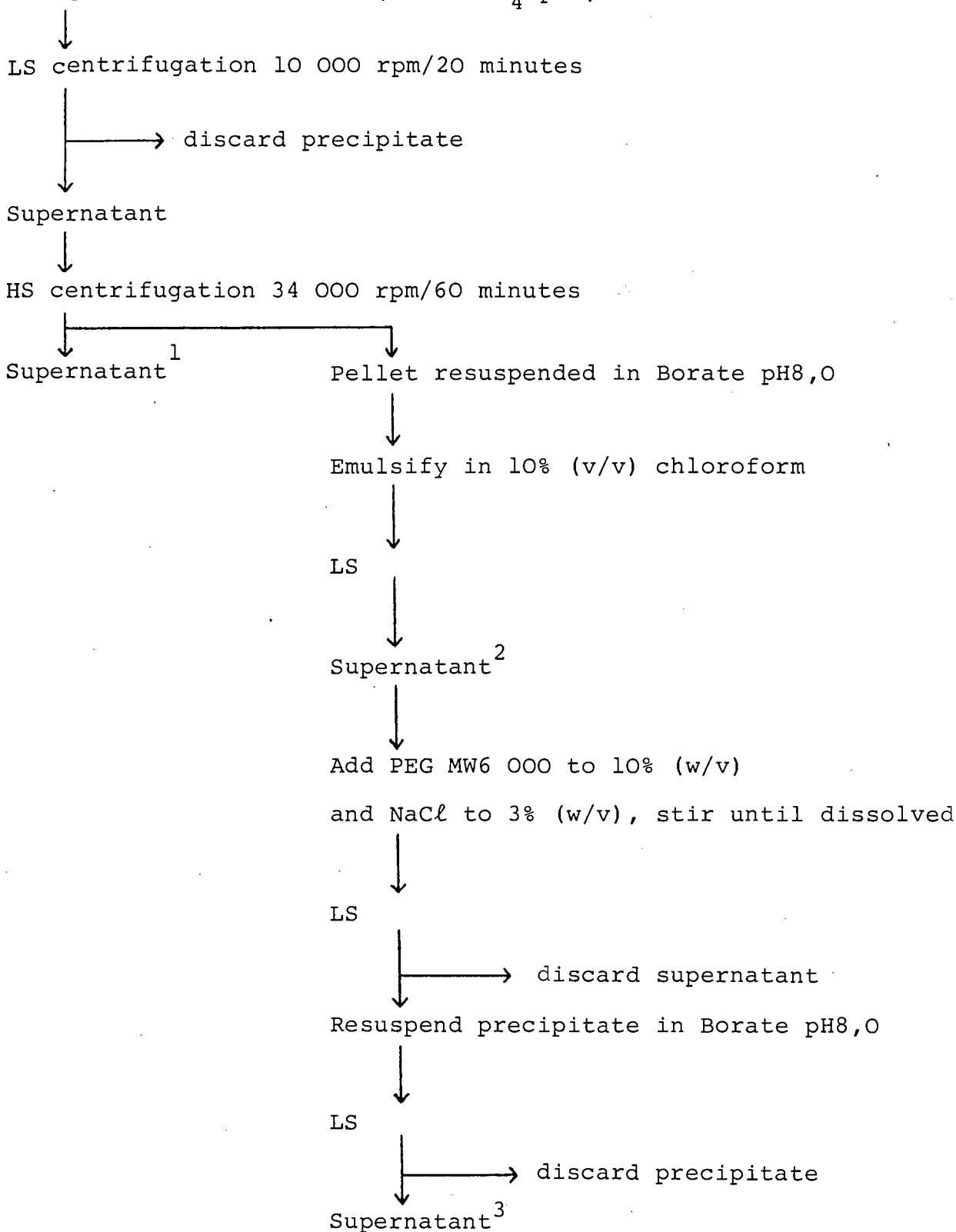
Uninoculated maize was processed by the purification method detailed in section III.C.2. The final extract was resuspended in borate pH8,0. Serial 2-fold dilutions of this preparation was tested against 2-fold dilutions of anti-MDMV-Win-1 IgG in the micro-precipitin test. A positive reaction with a titre of 1/16 for the 1st dilution of Ag was observed.

In a second test, maize plants were homogenized in either  $KPO_4$  pH7,0 or borate pH8,0 buffer and were clarified in 10% (v/v) chloroform. The preparations were absorbed with anti-maize antiserum (see sections III.D.1 and IV.G.4) at a 5:1 ratio to remove antibodies specific for maize protein. For details of absorption procedure, see III.D.3.a). When tested against anti-MDMV-Win-1 IgG in the micro-precipitin test, both antigen preparations showed a positive reaction (a flocculent precipitate) with this antiserum up to a titre of 1/32, indicating that MDMV antigens were present. A homologous test with the anti-maize serum was done, as a control. Maize clarified sap, absorbed with anti-MDMV-Win-1 IgG, was used as antigen.

Not all uninoculated maize plants showed the presence of seed-transmitted virus. Plants were processed as detailed below and were tested with anti-MDMV-Win-1 IgG in the micro-precipitin test.

Procedure

200g of maize leaves showing no mosaic symptoms were homogenized in 200ml of 0,05M NaPO<sub>4</sub> pH7,2 on ice.



The supernatant preparations at the different stages 1, 2 and 3 as indicated above, showed no reaction in the micro-precipitin tests.

These results indicate that only a percentage of uninoculated maize contained the seed transmitted virus.

#### Spontaneous mosaic symptoms

Fast germinating maize seeds were planted and observed to determine the frequency of spontaneous mosaic symptoms arising from these uninoculated maize plants. These observations were made at 3 separate times. Plants were observed for up to two weeks, to allow symptoms to develop, but the plants that developed mosaic symptoms could be detected visually at 5 - 7 days after planting of germinated seeds. The percentage of plants showing mosaic symptoms is given below.

Table 21 : The percentage of maize KEP-Wiplat plants that developed spontaneous mosaic symptoms recorded in 3 separate instances by visual determination

| No. of plants showing mosaic symptoms | % plants with mosaic symptoms |
|---------------------------------------|-------------------------------|
| Total no. of plants observed          |                               |
| 1/400                                 | 0,25                          |
| 7/304                                 | 2,3                           |
| 1/107                                 | 0,91                          |

The mosaic symptoms caused by the seed transmitted virus were indistinguishable to that caused by sap transmitted MDMV as illustrated in figure 2.

Two approaches were followed to test the hypothesis that virus was transmitted through seed. Firstly, the agent causing the spontaneous mosaic symptom, was propagated, isolated and partially characterized. Secondly, the individual maize coleoptiles were tested for the presence of virus.

## 2. PROPAGATION AND ISOLATION

The agent causing the spontaneous mosaic symptoms was transmissible by mechanical inoculation onto maize plants. Examination of leaf dip preparations of maize

leaves showing mosaic symptoms, showed the presence of virus-like filaments.

The isolate was purified after propagation in maize, using the method detailed in section III.C.2. This was clarification of the homogenate in 20% (v/v) chloroform, followed by precipitation with PEG MW 6000 and 1 cycle of differential ultracentrifugation. A UV scan similar to that in Fig. 3 (section IV.A.4) was obtained at this stage. The yield ranged from 56 - 119 mg/kg fresh leaf weight. The virus was infectious for 6 days when kept at 4°C in 0,05M borate pH8,0. Filamentous particles were observed on examination of this sample under the electron microscope.

The above results indicated that a virus was responsible for the spontaneous mosaic symptoms observed.

### 3. PARTIAL CHARACTERIZATION OF THE ISOLATE.

#### HOST RANGE OF THE SEED-TRANSMITTED ISOLATE.

This isolate was mechanically inoculated onto five monocotyledonous and three dicotyledonous hosts. Maize leaves showing mosaic symptoms were ground in a mortar and pestle in  $KPO_4$  pH7,0 buffer using

carborundum as an abrasive. Plant debris was removed by squeezing through cheesecloth, celite was added as an abrasive and the sap inoculated onto plants with cottonwool. The results are shown in Table 22.

Table 22 : The symptoms observed when the seed-transmitted isolate was inoculated onto monocotyledonous and dicotyledonous hosts

| <u>Host</u>                             | <u>Total No. of plants inoculated</u> | <u>No. of plants showing symptoms</u> | <u>Symptoms observed</u>      |
|-----------------------------------------|---------------------------------------|---------------------------------------|-------------------------------|
| Maize, KEP-Witplat                      | 8                                     | 7                                     | mosaic                        |
| Sweetcorn cv. Golden Bantam             | 29                                    | 24                                    | pale-green mosaic             |
| Johnsongrass                            | 1                                     | 0                                     | -                             |
| Sugarcane, grown from virus-free clones | 11                                    | 5                                     | mosaic                        |
| Sorghum cv. Ambercane                   | 40                                    | 40                                    | yellow-green mosaic           |
| Barley cv. Clipper                      | 30                                    | 15                                    | necrotic streaks <sup>1</sup> |
| <u>Chenopodium hybridum</u> L.          | 16                                    | 0                                     | -                             |
| <u>Chenopodium quinoa</u> L.            | 16                                    | 0                                     | -                             |
| Squash cv. Long white                   | 24                                    | 0                                     | -                             |

<sup>1</sup>=These plants were back inoculated onto maize cv. KEP-Witplat. 18 days post inoculation, 5/16 maize plants showed mosaic symptoms. This shows that the isolate was transmissible to barley. When the leaf dip

preparations of barley was examined under the electron microscope, no filamentous particles were observed. This may probably be due to a low concentration of the virus in the plants. The seed transmitted isolate did not infect barley when tested a second time.

#### Electron microscopy

Leaf dip preparations of maize showing spontaneous mosaic symptoms were examined under the electron microscope. Filamentous particles were observed. A partially purified preparation of MDMV-ST also revealed the presence of filamentous particles in negatively stained preparations.

ISEM assay with 1/1000 dilution of anti-MDMV-Win-1 antiserum, showed the large number of trapped filaments, Fig. 24. The antiserum was absorbed with a concentrated preparation of slow germinating maize plants. The approximate width of the filamentous particles was 12,2nm. (Accurate measurements not done). In addition to this, spherical particles with a diameter about 33-40nm were observed. Some of the particles had a centre penetrated by stain, which was approximately 16-20nm in diameter. These spheres are further discussed in section IV.D. (Isometric viruses in maize extracts).

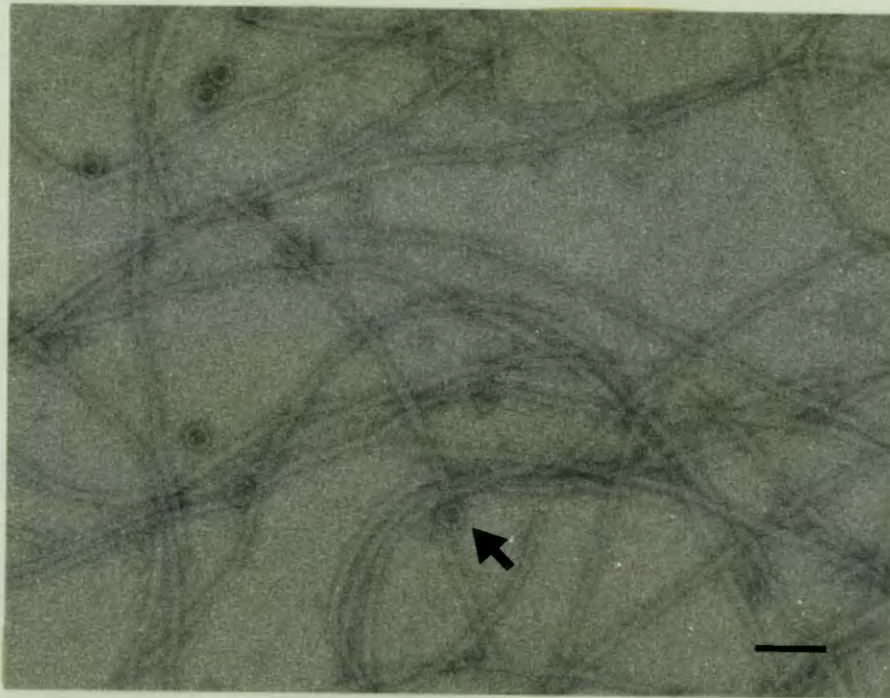


Figure 24 :

ISEM of MDMV-ST with anti-MDMV-Win-1 antiserum diluted at 1/1000. Antiserum was absorbed with a concentrated slow-germinating-maize preparation. Ab-trapped MDMV filaments as well as spherical particles were observed (the latter indicated by arrow). Magnification 90 000X. (The bar represents 100nm).

ELISA

Maize plants showing spontaneous mosaic symptoms were tested against anti-MDMV and anti-SCMV antisera in the sandwich ELISA test. Crude and concentrated plant sap was tested. Crude sap was prepared by grinding leaves in 1:1  $KPO_4$  buffer. Four-fold dilutions were tested. Concentrated preparations consisted of leaves ground in 2x volume  $KPO_4$  buffer. Plant debris was removed by LS centrifugation and the supernatant was centrifuged at 29 000 rpm for 90 minutes in a Beckman 30 rotor. The pellets were resuspended in borate pH8,0 in 1/10 the original volume of buffer. This was diluted serially 4-fold for testing. Both preparations reacted positively with anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-SCMV 4975-1 IgG as shown in Table 23.

Table 23 : The results obtained when the seed-transmitted virus was tested against antisera to MDMV-Win-1, MDMV-Kru-1 and SCMV 4975 isolates in the sandwich

ELISA.

| <u>Antisera</u>  | <u>Absorbance at 405nm</u>         |                      |                                           |                 |                                  |
|------------------|------------------------------------|----------------------|-------------------------------------------|-----------------|----------------------------------|
|                  | <u>Plant sap</u>                   |                      | <u>Concentrated preparations</u>          |                 |                                  |
|                  | <u>Spontaneous mosaic symptoms</u> | <u>Healthy maize</u> | <u>Seed transmitted virus<sub>1</sub></u> | <u>MDMV-Win</u> | <u>Healthy maize<sub>2</sub></u> |
| Anti-MDMV-Win-1  | 2,5                                | 0,157                | 2,5                                       | 2,5             | 0,235                            |
| Anti-MDMV-Kru-1  | 2,5                                | 0,068                | 2,5                                       | 2,5             | 0,082                            |
| Anti-SCMV 4975-1 | 2,8                                | 0,058                | 2,7                                       | 2,5             | 0,064                            |

1=Propagated from maize showing spontaneous mosaic symptoms.

2=Fast germinating maize, appearing healthy.

MDMV-ST (the seed-transmitted virus) was purified by the method detailed in section III.C. The virus was diluted 4-fold with a starting dilution of 2mg/ml.

Fig. 25 shows that MDMV-ST reacted positively with anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-SCMV 4975-1 IgG. The control, fast germinating maize, did not react with the antisera.

In a separate sandwich ELISA MDMV-ST was tested with anti-MDMV-A and anti-MDMV-B IgG, both kindly donated by D.T. Gordon, Ohio, U.S. MDMV-ST reacts positively with anti-MDMV-B IgG but not with anti-MDMV-A, Table 24.

Table 24 : The results obtained when MDMV-ST was tested against anti-MDMV-A and anti-MDMV-B IgG (U.S.) in the sandwich ELISA.

| <u>Antisera</u> | <u>Absorbance at 405nm</u> |                 |                          |
|-----------------|----------------------------|-----------------|--------------------------|
|                 | <u>MDMV-ST</u>             | <u>MDMV-Win</u> | <u>Healthy maize sap</u> |
| Anti-MDMV-A     | 0,028                      | 0,020           | 0,050                    |
| Anti-MDMV-B     | 2,7                        | 2,7             | 0,027                    |

The above results show that MDMV-ST is related to the local MDMV isolates and is an MDMV-B virus strain.

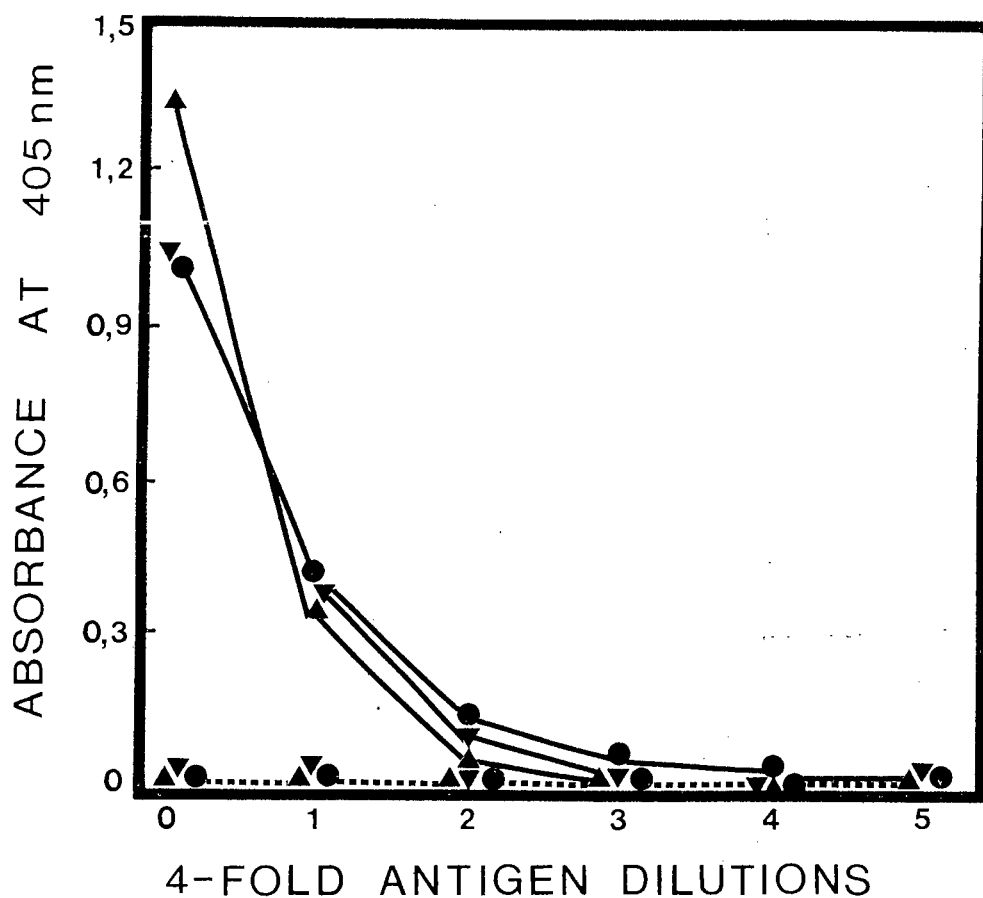


Figure 25 :

Sandwich ELISA test of MDMV-ST with anti-MDMV- and anti-SCMV-IgG's. The virus was diluted 4-fold starting with a concentrated preparation of fast germinating maize.

- = anti-MDMV-Kru-1
- ▲ = anti-MDMV-Win-1
- ▼ = anti-SCMV 4975-1
- = virus preparation
- ..... = control antigen

IEB assay

The seed-transmitted isolate (MDMV-ST), which was inoculated onto sorghum, sugarcane, barley and maize was tested in the IEB assay with various MDMV antisera.

The test samples were crude leaf extracts. Leaf samples showing symptoms were ground in a mortar and pestle.  $\text{KPO}_4$  pH7,0 buffer was added at 2:1 ratio to fresh leaf weight. The extract was squeezed through cheesecloth and centrifuged at LS. The sample was disrupted 1:1 with disruption mix for polyacrylamide gel electrophoresis (see III.G.1).

Figure 26 shows that MDMV-ST infected samples react with anti-MDMV-Win-1 (Fig. 26.a) and anti-MDMV-ST antisera (in Fig. 26.b and c) as a single component at the 36Kd position. The IgG's were used at 1/80 dilution. The MDMV-ST-infected sorghum sample reacted positively with both anti-MDMV-Win-1 IgG and anti-MDMV-ST IgG, as shown in lanes 2 of figures a and b respectively.

The barley samples in lane 4 Fig. 26.a and lane 3 Fig. 26.b respectively showed negative results with both antisera. Negative controls were uninoculated sorghum shown in lanes 1 of Fig. 26.a and b, and uninoculated barley samples in lane 5 Fig. 26.a and lane 4 Fig. 26.b. A positive control was MDMV-ST

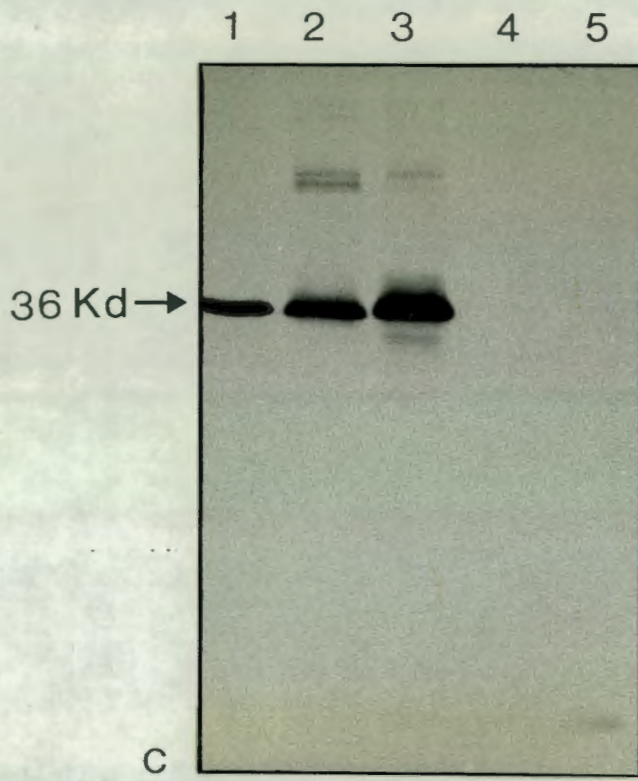
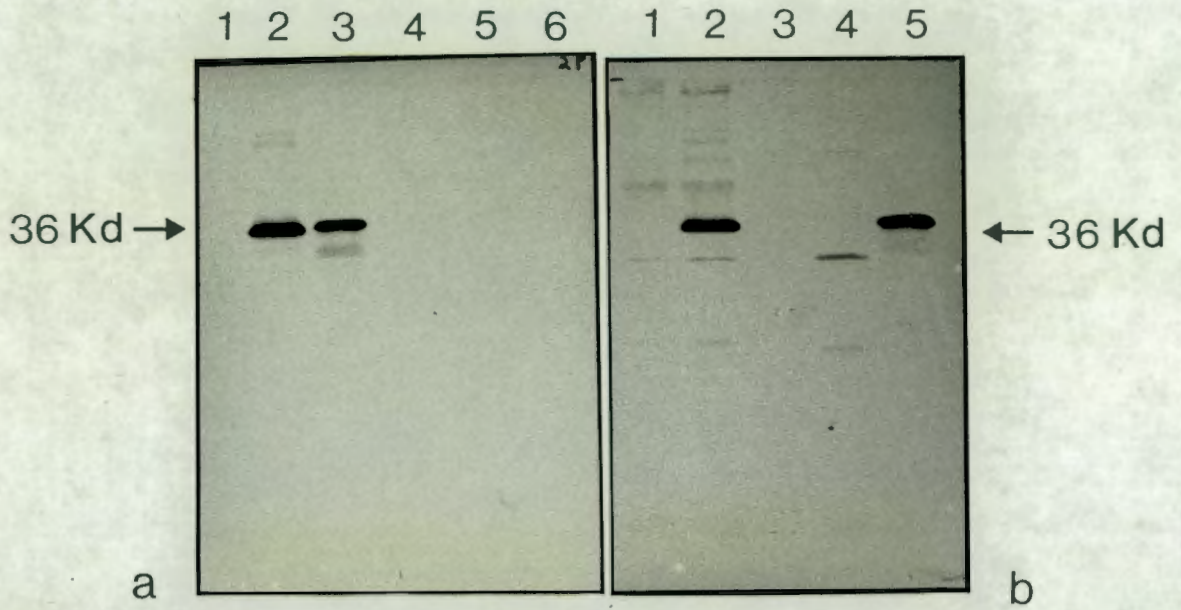


Figure 26 :

IEB assay of MDMV-ST inoculated onto different hosts and the reaction to anti-MDMV-ST and anti-MDMV-Win IgG.

a) and c) assayed with anti-MDMV-Win-1 IgG, 1/80 dilution

b) assayed with anti-MDMV-ST IgG, 1/80 dilution

The samples tested were crude sap extracts.

a, lane 1 = Uninoculated sorghum  
2 = MDMV-ST on sorghum  
3 = MDMV-ST on maize  
4 = MDMV-ST on barley  
5 and 6 = Uninoculated barley

b, lane 1 = Uninoculated sorghum  
2 = MDMV-ST on sorghum  
3 = MDMV-ST on barley  
4 = Uninoculated barley  
5 = MDMV-ST on maize

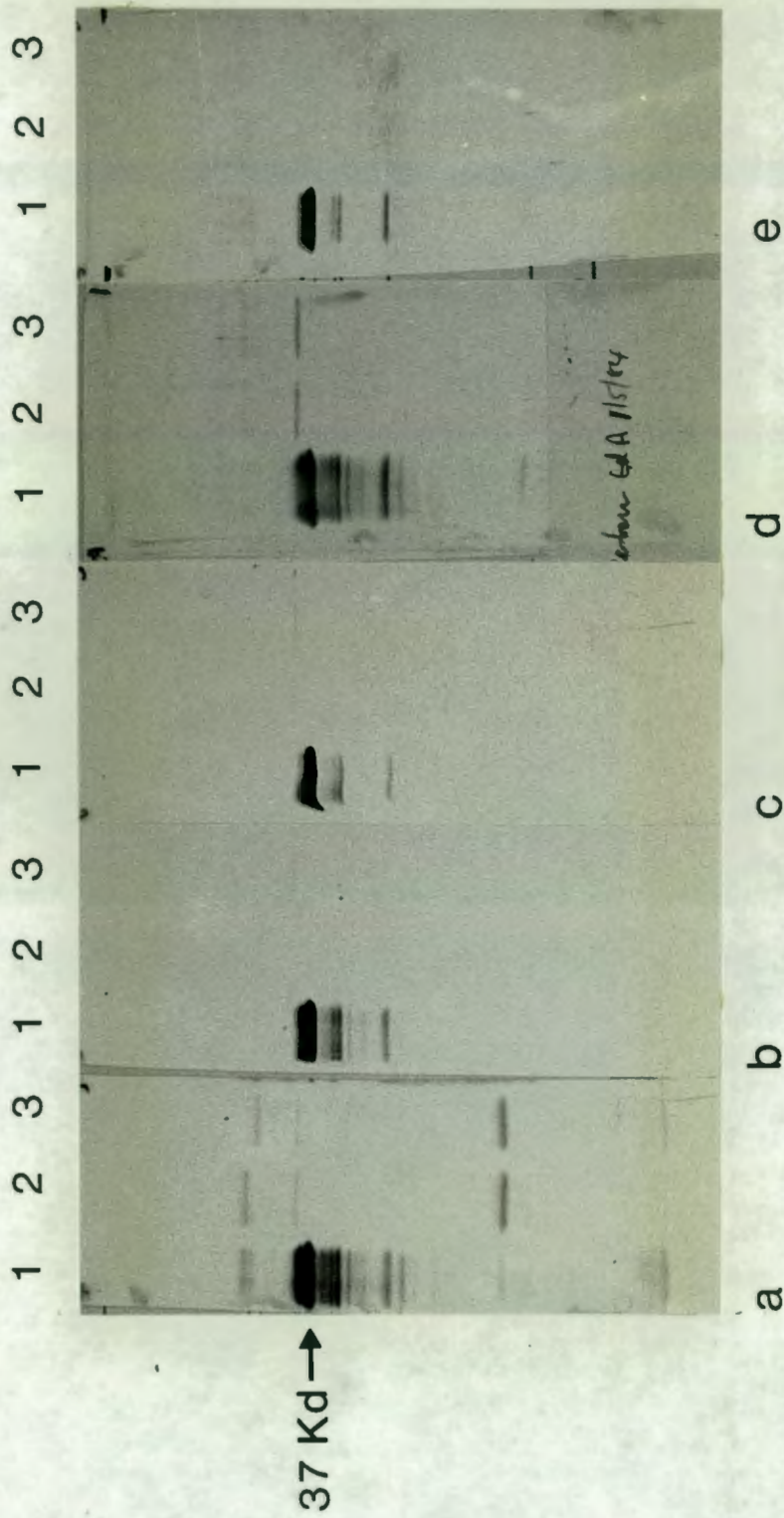
c, lane 1 = MDMV-ST on sugarcane  
2 = SCMV from infected sugarcane  
3 = MDMV-ST on maize  
4 = Uninoculated sugarcane  
5 = Uninoculated maize

inoculated on maize. This sample showed a single band at the 36Kd position when reacted with anti-MDMV-ST IgG as shown in lane 5, Fig. 26.b. Lane 3, Fig. 26.c shows the reaction with anti-MDMV-Win-1 IgG.

Sugarcane infected with MDMV-ST was tested with anti-MDMV-Win-1 IgG and the virus reacts as a single band as shown in lane 1 Fig. 26.c. Lanes 4 and 5, Fig. 26.c had samples of uninoculated sugarcane grown from virus-free plants and maize respectively. No reaction was observed. This sample was not tested with anti-MDMV-ST IgG.

SCMV that had been obtained from naturally infected sugarcane from Shongweni in Natal, showed a positive reaction, lane 2.c.

In contrast to the above, partially purified MDMV-ST obtained after 1 cycle of differential ultracentrifugation, was also tested with anti-MDMV antibodies in the IEB assay. A sample of 30ug protein was layered onto the polyacrylamide gels. Anti-MDMV-ST, anti-MDMV-Win-1 and anti-SCMV 4975 IgG were used at 1/80 dilution in Fig. 27.a, b and c. Anti-MDMV-Kru-1 antiserum diluted at 1/80 and anti-CMV antiserum (obtained from Mr. P. Lupuwana) diluted at 1/100 were host absorbed. MDMV-ST reacted positively with the



**Figure 27 :** IEB assay of MDMV-ST with various MDMV antisera and CMV antiserum.

The antisera/IgG used for the assay were:  
 a - anti-MDMV-ST IgG (1/80 dilution)  
 b - anti-MDMV-Win-1 IgG (1/80 dilution)  
 c - anti-SCMV 4975-1 IgG (1/80 dilution)  
 d - anti-MDMV-Kru-1 antiserum (1/80 dilution)  
 e - anti-CMV-1 antiserum (1/100 dilution)

Concentrated samples tested were: Lane 1 - MDMV-ST  
 Lane 2 - Virus-free maize grown from apical meristem tissue  
 Lane 3 - Uninoculated maize

antisera showing a major band at the 37Kd position. Several weaker bands at lower MW positions, i.e. at 34, 33 and 28Kd were observed in the reactions with the antibodies, in Fig. 27 a-e. These bands may be due to degradation of virus protein during the purification procedure and could be polypeptides recognized by the host absorbed antisera (IgG).

Concentrated host protein isolated from virus-free maize and uninoculated maize showed negative results with the MDMV and CMV antisera, lanes 2 and 3 respectively in Fig. 27 a-e.

CMV antiserum recognized MDMV-ST protein in Fig. 27.e, lane 1, at the same MW position, 37Kd, as the homologous reaction for MDMV-ST, which is shown in Fig. 27.a. This reaction was slightly less than with the homologous reaction but could not be due to a non-specific background reaction as it is specific for the polypeptide profile of MDMV. Reciprocal tests with CMV protein was therefore performed to test whether anti-MDMV antiserum reacted with CMV protein. Purified CMV, kindly donated by P. Lupuwana, was applied to wells at 15ug protein per sample. CMV reacted positively with the anti-CMV antiserum, lane 1 Fig. 28.a. The viral protein is at 24,4Kd position and the multiple bands above this are host protein bands. Anti-MDMV-Win-1

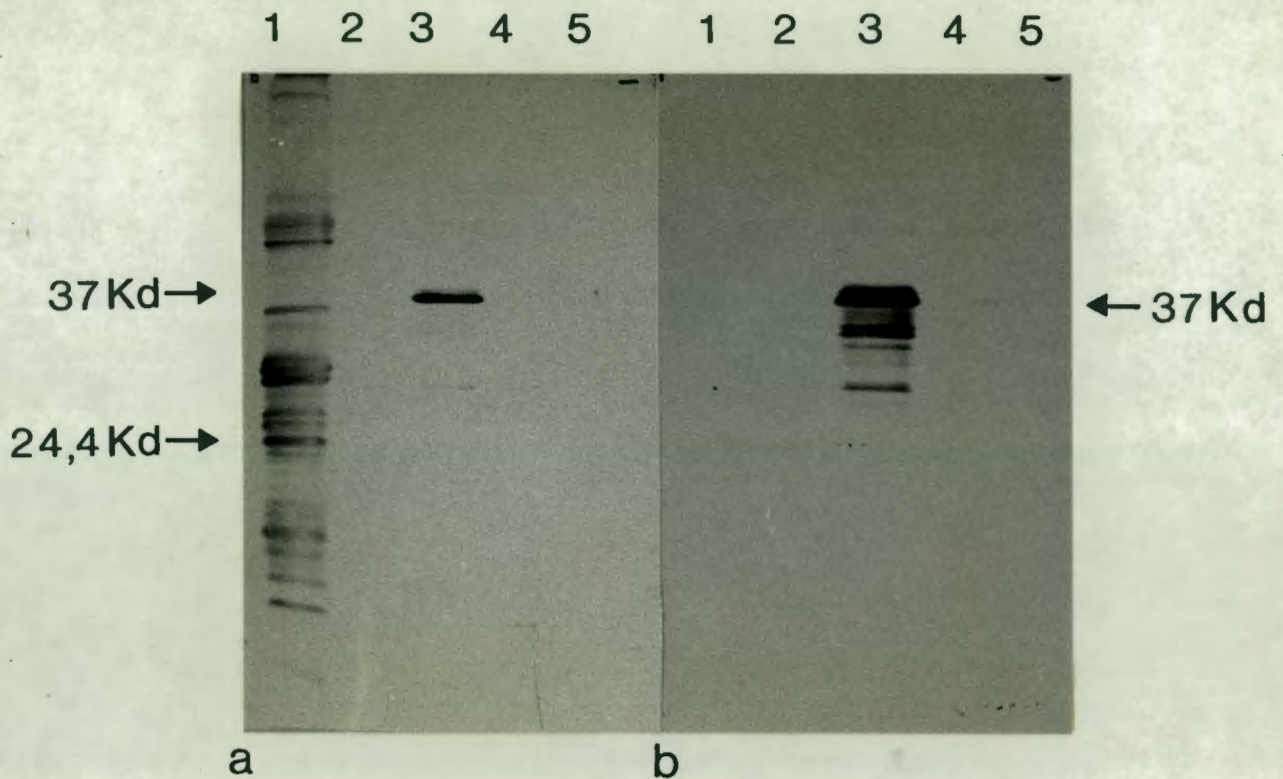


Figure 28 :

IEB assay of MDMV-ST tested with anti-CMV-1 antiserum and anti-MDMV-Win-1 IgG.

a - assayed with anti-CMV-1 (not host absorbed), 1/100 dilution

b - assayed with anti-MDMV-Win-1 IgG, 1/80 dilution

Purified samples tested were:

- Lane 1 - CMV
- 2 - No sample
- 3 - MDMV-ST
- 4 - Virus-free maize grown from tissue culture
- 5 - Uninoculated maize

did not react with CMV, lane 1 Fig. 28.b. MDMV-ST was recognised by anti-MDMV-Win-1 IgG, lane 3 Fig. 28.b and also by anti-CMV antiserum lane 3 Fig. 28.a but less strongly. The maize controls were negative.

The reaction of CMV with host absorbed anti-CMV-1 and CMV-2 is shown in Fig. 29.a and b respectively. (Figure reproduced from P. Lupuwana, 1985). Lanes 1, 2 and 3 contained CMV isolate-1, -2 and -3 respectively. The CMV protein migrated to the 24,5Kd position. The healthy tobacco control, which showed no reaction, is in lane 4.

The cross reactivity of MDMV with anti-CMV-serum is not understood. I did not pursue this aspect, but it is of interest that a similar observation was made by A. Milligan when he investigated a natural double infection of MDMV and CMV in maize originating in Natal (BSc. Hons. project, U.C.T., 1984). See also section IV.E, on Rhooodeplaat isolates.

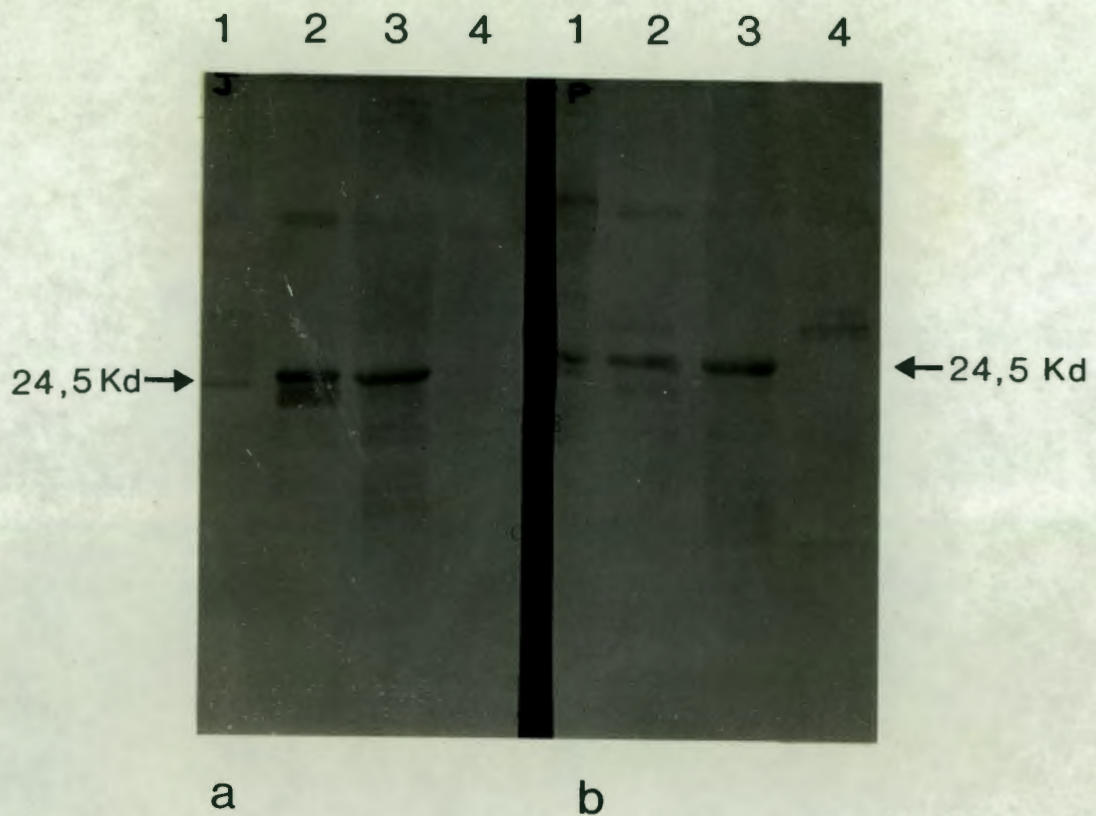


Figure 29 :

An IEB assay to show the reactions of host absorbed CMV anti-sera.

a - assayed with anti-CMV-1 antiserum

b - assayed with anti-CMV-2 antiserum

Samples are:

Lane 1 - CMV isolate-1

2 - CMV isolate-2

3 - CMV isolate-3

4 - Healthy tobacco

(Figure 29, a and b reproduced with kind permission from P. Lupuwana, 1985).

4. THE TESTING OF SEED COLEOPTILES FOR THE PRESENCE OF SEED TRANSMITTED VIRUS.

Maize seeds were randomly picked from the seed supply and were pregerminated on moist cottonwool for 3-5 days at 25°C. All the embryos did not grow at the same rate. The seeds were divided into fast and slow germinating categories, according to the length of shoots and roots, if the latter was also present. A sample of germinated seedlings is shown in Fig. 30 and the percentage of fast and slow germinating seeds is shown in Table 25.

Table 25 : Maize cv KEP-Witplat seeds, germinated for 3-5 days, showing the percentage of fast and slow germinating seeds.

| <u>Category</u>                        | <u>Number</u> | <u>%</u> |
|----------------------------------------|---------------|----------|
| <u>Fast germinating seeds</u>          |               |          |
| 1. Shoots up to 2-4cm, roots present   | 74            | 36,3     |
| <u>Slow germinating seeds</u>          |               |          |
| 2. Shoots up to 1-2cm, roots present   | 82            | 40,2     |
| 3. Shoots just emerging, roots present | 20            | 9,8      |
| 4. Shoots not present, roots present   | 14            | 6,86     |
| 5. No shoots, no roots                 | 14            | 6,86     |
| Total                                  | 204           |          |

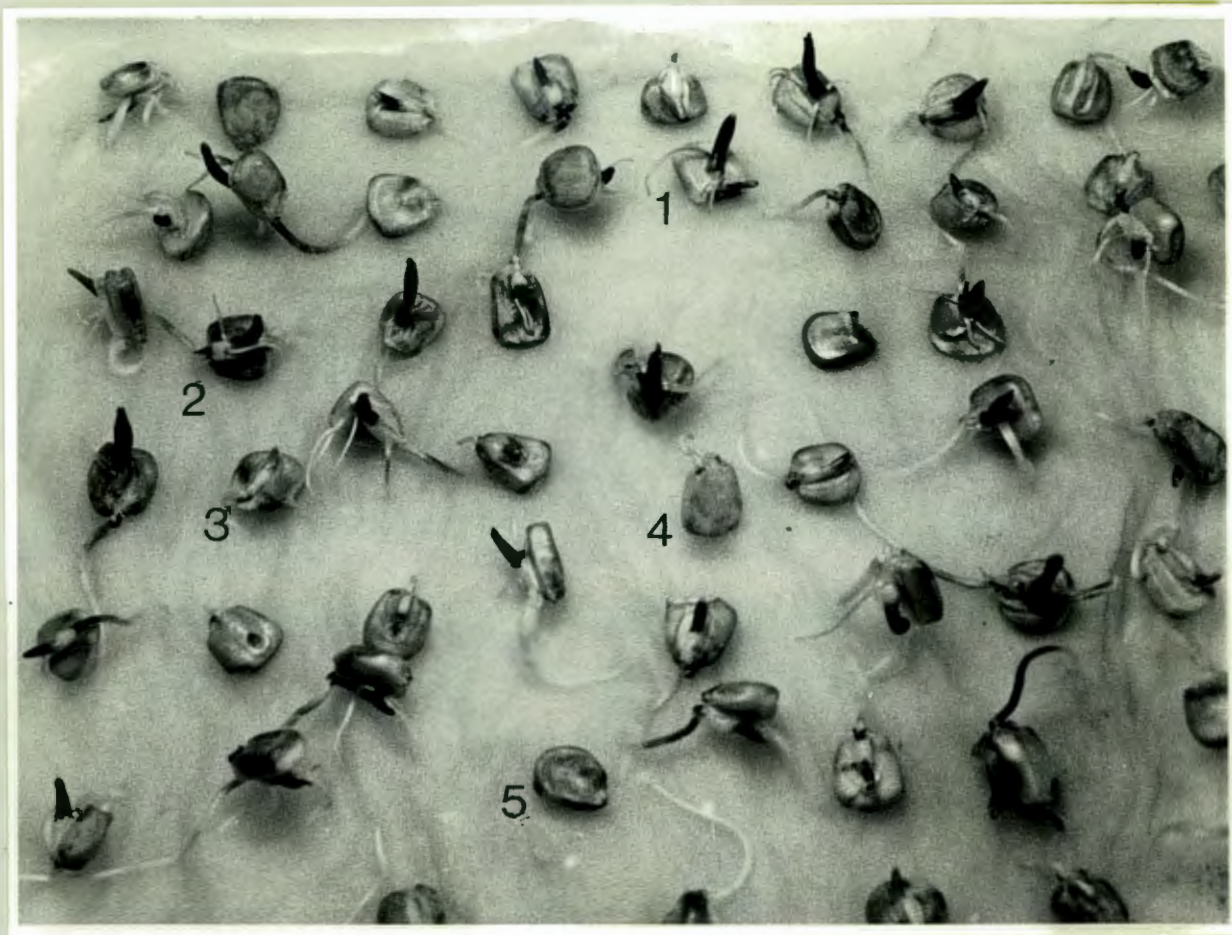


Figure 30 : Germinating maize seed after 3 days incubation, showing the different rates of growth of the embryos.

- 1 - Fast germinating seed, shoots up to 2 - 4cm, roots present
- 2 - Slow germinating seed, shoots up to 1 - 2cm, roots present
- 3 - Slow germinating seed, shoots just emerging, roots present
- 4 - Slow germinating seed, shoots not present, roots present
- 5 - Slow germinating seed, no shoots, no roots.

36% of the seeds were fast germinating, showing that the majority of the coleoptiles were retarded in growth, i.e. slow germinating.

Individual embryos from different categories were excised and crushed in a mortar and pestle in 2ml  $KPO_4$  pH7,0 buffer. Two-fold dilutions of the extracts were tested in the sandwich ELISA test with anti-MDMV-Kru-1 and anti-SCMV 4975-1 IgG. Some of the extracts were also tested with anti-MDMV-A and anti-MDMV-B IgG obtained from D.T. Gordon, Ohio, U.S.

Fast germinating embryos showed relatively lower absorbance readings than the slow germinating embryos in the ELISA, and these were regarded as negative readings, see Table 26. With both anti-MDMV-Kru-1 and anti-SCMV 4975-1 IgG, 7 out of the 14 slow germinating embryos showed a positive reaction. This shows that the slow germinating embryos contained virus antigen. Fig. 31 shows the curves obtained in one of the sandwich ELISA tests with anti-SCMV 4975 IgG.

A small number of fast and slow germinating embryos were tested with anti-MDMV-A and anti-MDMV-B IgG (both obtained from D.T. Gordon, United States) as a limited amount of

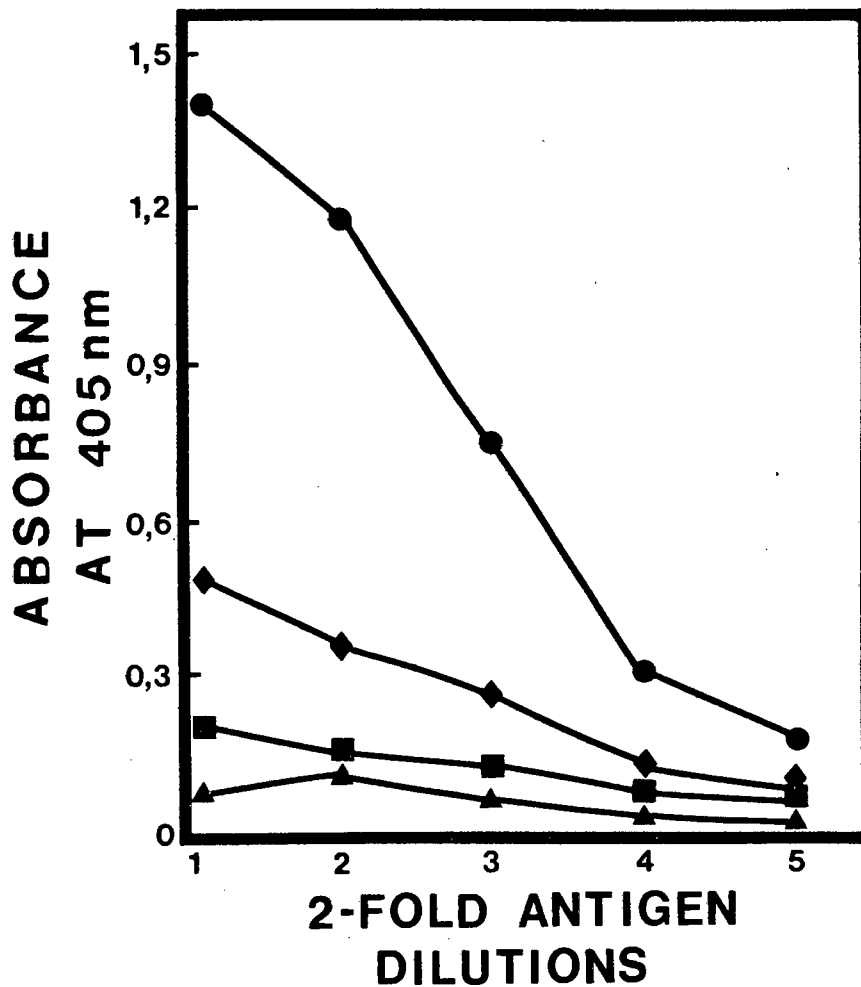


Figure 31 : Sandwich ELISA with anti-SCMV 4975-1 to test for MDMV in maize coleoptiles. Individual coleoptiles were excised and crushed in 2ml 0,1M  $KPO_4$  pH 7,0 in a mortar and pestle.

- ▲ represents fast germinating embryos (refer Table 25 and Fig. 30,1)
- represents slow germinating embryos (refer Table 25 and Fig. 30,2)
- represents slow germinating embryos (refer Table 25 and Fig. 30,3)
- ◆ represents slow germinating embryos (refer Table 25 and Fig. 30,5)

serum was available. Both the fast and slow germinating embryos failed to react with these anti-sera, (Table 26). The negative control used was uninoculated maize sap and the positive control was purified MDMV-ST.

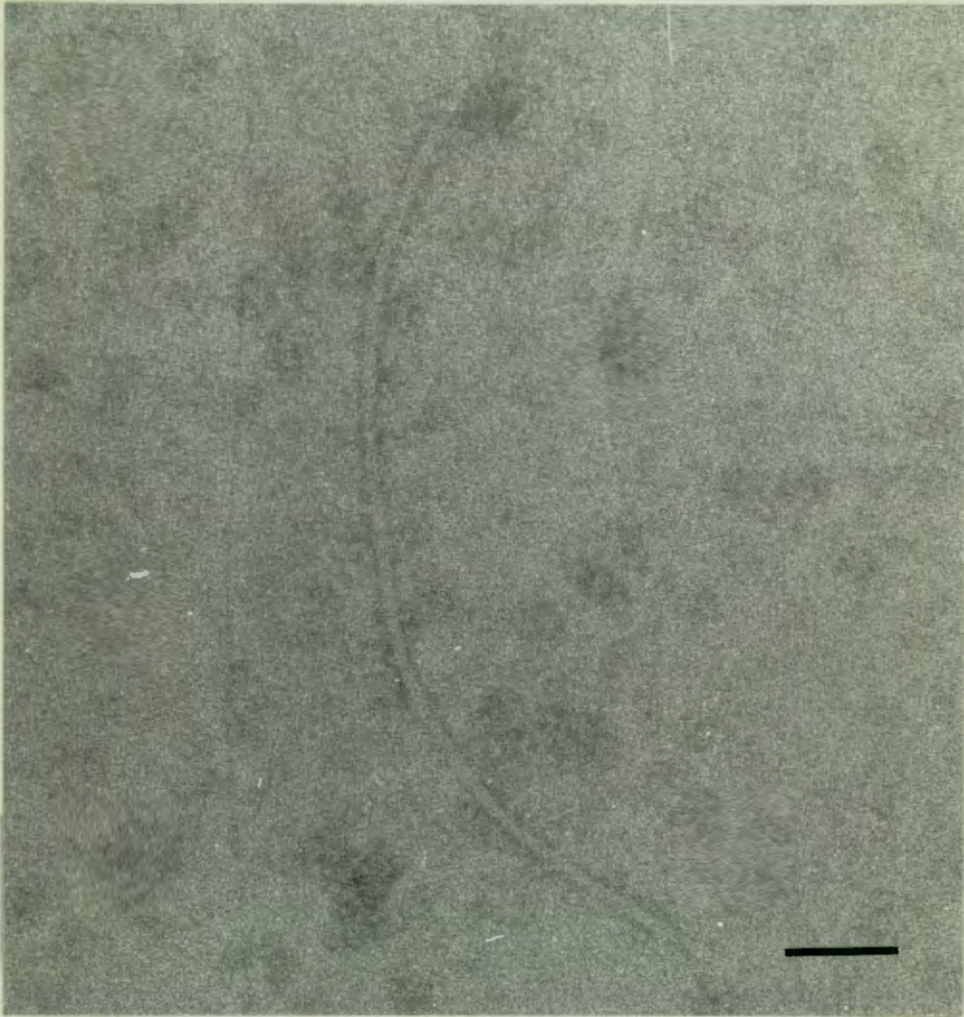


Figure 32 : Electron micrograph of a concentrated preparation of apparently healthy maize. The stain was 2% PTA, pH 7,0. Magnification was 150 000 x. Bar represents 100nm.

Table 26 : The results when individual fast and slow germinating maize embryos were tested with anti-MDMV-Kru-1 and anti-SCMV 4975 IgG and anti-MDMV-A and anti-MDMV-B IgG in the sandwich ELISA.

| <u>Test Antigen</u>           |    | <u>Absorbance at 405nm</u> |                        |                    |                    |
|-------------------------------|----|----------------------------|------------------------|--------------------|--------------------|
| <u>Embryo samples</u>         |    | <u>anti-SCMV 4975</u>      | <u>anti-MDMV-Kru-1</u> | <u>anti-MDMV-A</u> | <u>anti-MDMV-B</u> |
| <u>Fast germinating seeds</u> |    |                            |                        |                    |                    |
| Shoots up to 2-4cm            | 1  | 0,125-                     | 0,202- <sup>2</sup>    | x                  | 0,028-             |
|                               | 2  | 0,135-                     | 0,179-                 | x                  | 0,016-             |
|                               | 3  | 0,183-                     | x <sup>3</sup>         | x                  | x                  |
|                               | 4  | 0,117                      | x                      | x                  | x                  |
|                               | 5  | x                          | 0,166-                 | 0,027-             | x                  |
|                               | 6  | x                          | 0,240-                 | x                  | x                  |
| <u>Slow germinating seeds</u> |    |                            |                        |                    |                    |
| Shoots up to 1-2cm            | 7  | 0,457+                     | 0,528+ <sup>1</sup>    | x                  | 0,048-             |
|                               | 8  | 0,214-                     | x                      | x                  | x                  |
|                               | 9  | x                          | 0,195-                 | 0,060-             | x                  |
|                               | 10 | x                          | 0,539+                 | x                  | x                  |

|                                       |    |        |        |        |        |
|---------------------------------------|----|--------|--------|--------|--------|
| Shoots just emerging                  | 11 | 0,594+ | 0,175- | x      | 0,023- |
|                                       | 12 | x      | 1,407+ | x      | x      |
|                                       | 13 | x      | 0,438+ | x      | x      |
|                                       | 14 | 0,155- | x      | 0,024- | x      |
|                                       | 15 | 0,187- | x      | x      | x      |
| No shoots, no roots                   | 16 | 0,123- | 0,127- | x      | 0,004- |
|                                       | 17 | x      | 0,472+ | x      | x      |
| <u>Controls</u>                       |    |        |        |        |        |
| MDMV-ST                               |    | 2,7+   | 2,7+   | 0,028- | 2,7+   |
| Sap from uninoculated<br>maize plants |    | 0,086- | 0,095- | 0050-  | 0,027- |
| "                                     |    | 0,157- | 0,139- | x      | x      |

1=positive result.

2=negative result.

3=Not tested.

## 5. PRESENCE OF VIRUS IN SYMPTOMLESS PLANTS

Healthy appearing maize plants were also tested for the presence of MDMV. These plants did not show any mosaic symptoms. Four to five week old plants grown from slow germinating and fast germinating maize were examined. This was the same age that inoculated plants were normally harvested.

These plant extracts were concentrated by differential ultracentrifugation and were examined under the electron microscope. The electron micrograph, Fig. 32, illustrates one of a few virus-like filaments obtained from a concentrated preparation of fast germinating maize. A filamentous particle was also observed in the slow germinating maize preparation.

In the ISEM assay, with anti-MDMV-Win-1 antiserum, no virus particles were trapped. These preparations were also tested in the sandwich ELISA test. A control preparation of sap from maize grown from apical meristem tissue was used. Both the slow and fast germinating maize preparations showed positive results when reacted with anti-MDMV-Kru-1 IgG as shown in Fig. 33. The control maize preparation (maize grown from apical meristem tissue) did not react with the anti-MDMV-Kru-1 IgG.

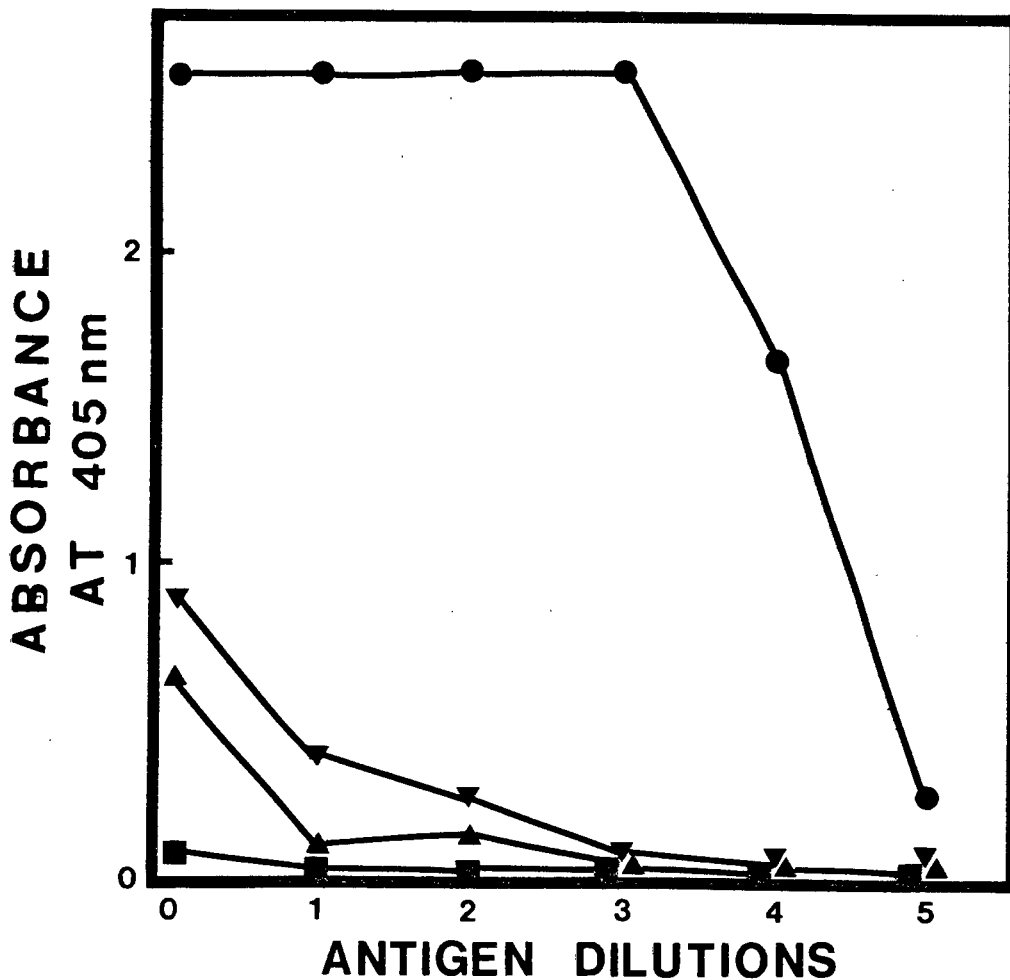


Figure 33 : The reactions of fast and slow germinating maize extracts in the sandwich ELISA test with anti-MDMV-Kru-1 IgG. The coating antibody and the alkaline phosphatase conjugated IgG was used at 1/400 dilutions.

- ▼ = 4-fold dilutions of concentrated extracts of symptomless fast germinating maize
- ▲ = 4-fold dilutions of concentrated extracts of symptomless slow germinating maize
- = 2-fold dilutions of sap of maize grown from apical meristem tissue culture
- = 4-fold dilutions of MDMV-ST from spontaneous mosaic symptom-producing plants

In another test where an extract of symptomless fast germinating maize was tested in sandwich ELISA against 3 different antisera i.e. anti-MDMV-Win-1, anti-MDMV-Kru-1 and SCMV 4975-1 no reaction was recorded (See Fig. 25). A possible explanation for this discrepancy is that seedborne virus is present only in a certain percentage of seed and that it is possible to have batches of plants (from the same seed source) that are free of virus. (See Table 26).

#### 6. SEED TRANSMISSION IN OTHER MAIZE LINES

The fact that a seed transmitted MDMV was found in maize cv. K.E.P.-Witplat (a discontinued commercial line), prompted the investigation for the possible presence of seed transmitted MDMV in other maize lines. These were maize cv. Goudveld, and two maize hybrids, No.1 and No.2.

##### (a) Goudveld

The seed of a maize cultivar, Goudveld (old commercial cv.) was examined for seed transmitted virus. About 200 randomly picked seed were pre-germinated on moist cottonwool for three days. The seeds germinated at different rates and were grouped according to the size of coleoptiles present. The percentage of seed in each category is shown in Table 27.

Table 27 : The percentage of fast and slow germinating seed of maize cv. Goudveld (after 3 days of germination).

| <u>Category</u>                      | <u>Number</u> | <u>%</u> |
|--------------------------------------|---------------|----------|
| <u>Fast germinating seed</u>         |               |          |
| 1. Shoots up to 1,5cm, roots present | 41            | 19,8     |
| <u>Slow germinating seed</u>         |               |          |
| 2. Shoots up to 1cm, roots present   | 73            | 35,3     |
| 3. Shoots up to 0,5cm, roots present | 50            | 24,2     |
| 4. No shoots, roots present          | 32            | 15,5     |
| 5. No shoots, no roots               | 11            | 5,3      |
|                                      | 207           |          |

19,8% of the 207 seeds examined were fast germinating and 5,3% failed to germinate and the majority (75%) were slow germinating seeds.

Individual embryos from each category were tested for the presence of MDMV in the ELISA test. Each embryo was excised and ground in 2ml  $KPO_4$  pH 7,0 in a mortar and pestle and two-fold dilutions were made. The results are shown in Table 28.

Table 28 : Sandwich ELISA of embryos of maize cv.

Goudveld tested with anti-MDMV-Kru-1 IgG.

| <u>Antigen</u>                    | <u>Embryo number</u> | <u>Absorbance at 405nm</u> | <u>Rating</u> |
|-----------------------------------|----------------------|----------------------------|---------------|
| <u>Embryo samples</u>             |                      |                            |               |
| <u>Fast germinating seed</u>      |                      |                            |               |
| Shoots up to 1,5cm, roots present | 1                    | 0,374                      | +             |
|                                   | 2                    | 0,259                      | -             |
|                                   | 3                    | 0,300                      | -             |
|                                   | 4                    | 0,336                      | +             |
| <u>Slow germinating seed</u>      |                      |                            |               |
| Shoots up to 1cm, roots present   | 5                    | 0,316                      | +             |
|                                   | 6                    | 0,502                      | +             |
|                                   | 7                    | 0,602                      | +             |
| Shoots up to 0,5cm, roots present | 8                    | 0,391                      | +             |
|                                   | 9                    | 0,477                      | +             |
|                                   | 10                   | 0,686                      | +             |
| No shoots, roots present          | 11                   | 1,973                      | +             |
| No shoots, no roots               | 12                   | 0,616                      | +             |
| <u>Controls</u>                   |                      |                            |               |
| Uninoculated maize sap            |                      | 0,132                      | -             |
| Uninoculated maize sap            |                      | 0,145                      | -             |
| Sap of maize (cv. KEP-Witplat)    |                      | 0,092                      | -             |
| Grown by tissue culture           |                      | 0,114                      | -             |
| MDMV-ST (0,2mg/ml)                |                      | 2,9                        | +             |

An absorbance reading, twice that of the uninoculated maize sap control ( $Abs_{405} = 0,145$ ) and rounded off to  $Abs_{405} = 0,300$ , was taken as a negative result. Two of the four fast germinating embryos tested, showed a positive reading. Of the eight slow germinating embryos tested, seven were positive and one sample showed a reading close to the negative threshold of  $Abs_{405} = 0,300$ . The embryos contained virus in the range 0,78 - 3,1ug/ml virus/coleoptile as estimated from a concentration/absorbance graph for MDMV-ST. Of the two-fold dilutions of the coleoptile extracts tested in ELISA, the dilutions of six out of twelve extracts showed higher absorbance readings than the undiluted extracts. It is possible that the host proteins in the undiluted extracts caused steric hindrance of virus attachment to the antibodies and therefore showed lower absorbance values than the diluted samples.

Germinated coleoptiles from the fast and slow germinating categories were planted out after one week at 4°C and transferred to the plant growth room. No mosaic symptoms were observed after one week, but one of the slow germinating plants exhibited a 'dead growing point' symptom.

Three out of sixteen plants had developed spontaneous mosaic symptoms. Sap of the leaves with the spontaneous mosaic symptoms were electrophoresed on SDS-PAGE gels.

The control sample was MDMV-ST infected maize cv. K.E.P.-Witplat. Both these samples contained protein bands at the 40Kd and 34Kd positions, which were not present in an uninfected maize control, which was sap of maize cv. K.E.P.-Witplat grown from apical meristem tissue (and believed to be virus free). This indicated that MDMV-ST and the virus causing the spontaneous mosaic symptoms in maize cv. Goudveld may be the same. Further investigation with ELISA and IEB assays are required to establish conclusively the presence of the seed transmitted virus in maize cv. Goudveld as indicated by these preliminary tests.

(b) Maize hybrids

Having found seedborne-MDMV in K.E.P.-Witplat and Goudveld maize, which were old commercial cultivars (no longer planted commercially), it was of interest to establish whether contemporary maize hybrids were free of MDMV.

Four random hybrids were checked and two of them were found to contain MDMV as reported below. The identity of the hybrids is not given as the sample examined is not representative of all the hybrids available commercially and the limited examination may give a biased impression.

(b) (i) Maize hybrid No.1

Approximately 2 - 4% seedlings of uninoculated maize hybrid No.1 exhibited spontaneous mosaic symptoms. The agent causing these symptoms was mechanically transmissible. Maize cv. K.E.P.-Witplat was inoculated and developed mosaic symptoms. The fast germinating seedlings of K.E.P.-Witplat maize were used as a propagation host.

Filamentous virus-like particles were observed in the electron microscope, in leaf dip preparations of maize showing mosaic symptoms. An ISEM assay of this preparation using 1/1000 dilution of anti-MDMV-Win-1 IgG, trapped filamentous particles (Fig. 34). This result indicated that a filamentous virus antigenically related to MDMV-Win was responsible for the spontaneous mosaic symptoms in maize hybrid No.1.

Virus was purified using the method described in section III.C.2. The yield was 30,9mg virus/kg fresh leaf weight. Purification was also attempted using the method of Seghal (1968) which is given overleaf.

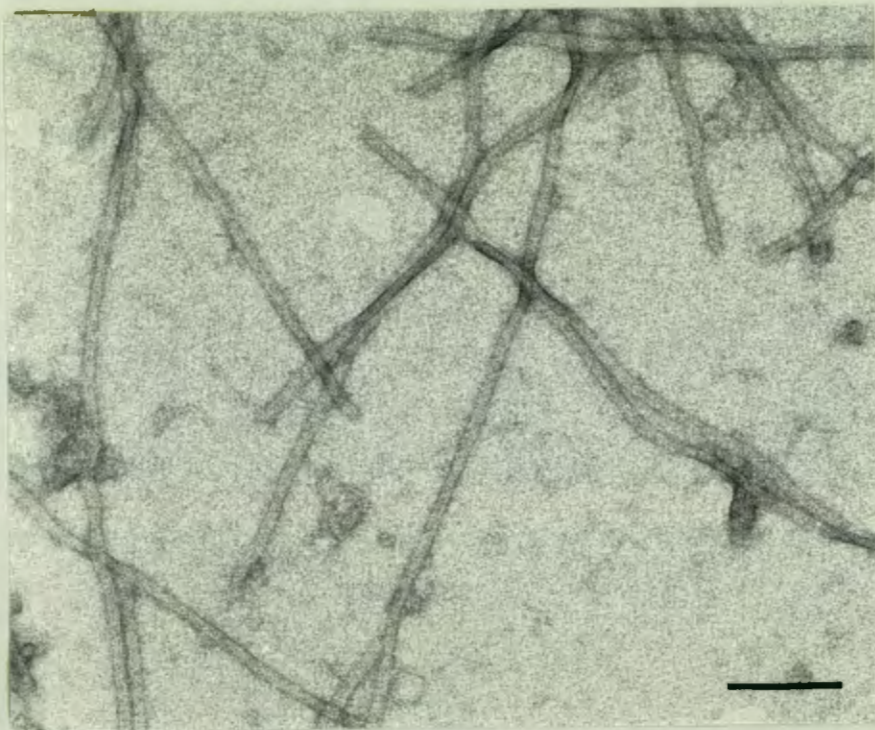


Figure 34 : ISEM of maize hybrid No.1 virus (purified preparation) trapped with anti-MDMV-Win-1 IgG (1/1000 dilution). The magnification was 150 000 x. The bar represents 100nm.

Infected leaf tissue homogenized in 4 volumes  
67mM  $KPO_4$  pH 7,0

↓  
Strain through cheesecloth

↓  
LS 8000rpm/20 minutes

→ Discard precipitate

↓  
Adjust supernatant to pH 5,3 by adding IN HCl  
while stirring

↓  
Leave in ice bath for 30 minutes

↓  
LS 3000rpm/20 minutes

→ Discard precipitate

↓  
Add DEAE cellulose powder, 5g/100ml supernatant,  
allow to remain for 20 minutes

↓  
Filter through Buchner funnel

↓  
Filtrate, HS 30 000rpm/60 minutes

→ Discard supernatant

↓  
Resuspend pellet in 0,05M borate pH 7,6

↓  
LS 3000rpm/20 minutes

→ Discard precipitate

↓  
Supernatant

The yield was 15mg virus/kg fresh leaf weight.

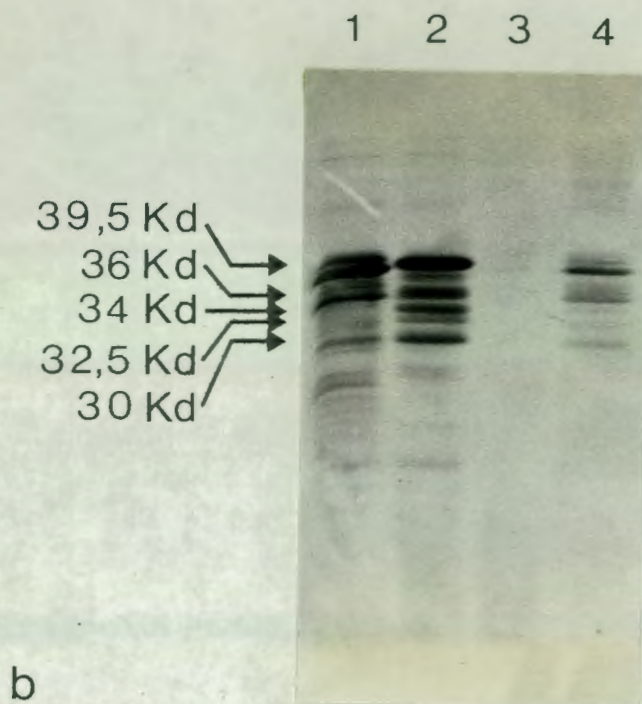
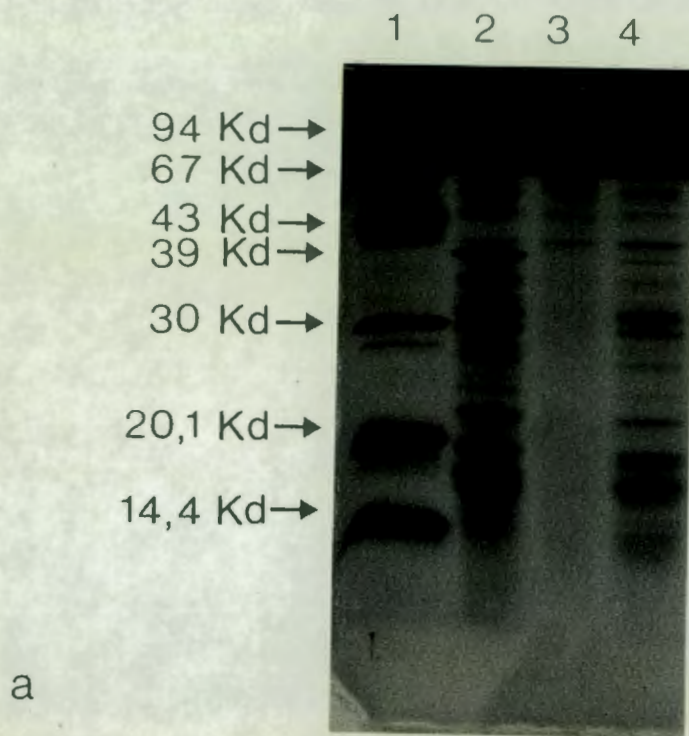
Maize hybrid-1 virus reacted with anti-MDMV-Win-1 IgG up to a titre of 1/32 in the microprecipitin test.

Maize hybrid No.1 virus was electrophoresed on SDS-polyacrylamide gels. Protein bands at 39Kd, 36Kd and 32,5Kd positions were observed (Fig. 35a). Fig. 35b shows the results of an IEB assay using anti-MDMV-Win-1 IgG (1/30 dilution) for the detection of MDMV. A strong positive reaction was observed for maize hybrid No.1 virus (Fig. 35b, lane 2). The protein bands at molecular weights of 39,5Kd, 36Kd, 34Kd, 32,5Kd, 30Kd were detected. The polypeptide profile of maize hybrid No.1 virus is different to that of MDMV-ST (from K.E.P.-Witplát) (Fig. 35b, lanes 1 and 4), but the major bands are at the 39Kd position.

(b) (ii) Maize hybrid No.2

This was another maize hybrid examined which exhibited spontaneous mosaic symptoms on uninoculated maize seedlings. The agent was mechanically transmissible to maize cv. K.E.P.-Witplat, as these plants became infected showing mosaic symptoms. Electron micrographs of leaf dip preparations showed the presence of filamentous particles.

The maize hybrid No.2 virus was propagated on K.E.P.-



**Figure 35 :** (a) SDS-PAGE of seed-transmitted virus from maize cv. KEP-Witplat and maize hybrids No.1 and No.2. The samples were partially purified preparations. (b) IEB assay of the above samples, tested for MDMV with anti-MDMV-Win-1 IgG (1/30 dilution). The samples were: a, (1)Molecular weight markers (Pharmacia) b,(1) MDMV-ST from maize cv. KEP-Witplat (2) Maize hybrid No.1 (3) Maize hybrid No.2 (4) MDMV-ST from maize cv. KEP-Witplat. (2, 3 and 4 are the same in a and b).

Witplat maize and purified using the method outlined in section III.C.2. This was not a successful purification procedure as electron microscopic examination of the final product did not show any filamentous particles.

ISEM assays with anti-MDMV-Win-1, anti-MDMV-Kru-1, anti-MDMV-CMV-1 antisera were negative, as was the IEB test with anti-MDMV-Win-1 IgG. This was ascribed to poor preparation of virus.

The above tests show that maize hybrid No.1 contained a MDMV- isolate that is strongly related, if not identical to the MDMV-ST and MDMV-Win isolates and is of the MDMV-B strain. The negative result obtained with maize hybrid No.2 could have two possible explanations:

- (i) that the virus was lost during preparation, or
- (ii) that the MDMV-A strain was involved.

#### D. APHID TRANSMISSION STUDIES

MDMV is known to be non-persistently transmitted by a large number (over 20) of different aphid species (Knoke and Louie, 1981). Most of the aphid genera are found in the sub-family Aphidinae (Nault and Knoke, 1981)

of which Rhopalosiphum is one.

Aphid transmission tests were attempted with two aphid species, Rhopalosiphum padi (L) and Diuraphis noxia (one of five aphids found on wheat and found to transmit Free State Streak Disease (von Wechmar and Rybicki, 1981 ). The other species, R. padi, R. maidis, Schizaphis graminum and Myzus persicae are all known vectors of MDMV. Transmission studies were done with MDMV-ST.

#### PROCEDURE

Clean (virus-free clone) colonies of D. noxia and R. padi (obtained from U.C.T. aphid collection, maintained on barley cv. Clipper) were transferred to fast germinating maize plants which had previously been sap inoculated with MDMV-ST. Aphids were allowed to feed on these source plants in a cage for one day. The aphids were then transferred to uninfected fast germinating maize, or in some cases, to maize grown from apical meristem tissue. The aphids were allowed to feed for 1-3 days before being sprayed with insecticide.

Aphid-inoculated plants were allowed to grow for 2-4 weeks. Plants were examined for the presence of virus by visual assessment of symptoms and by testing extracts of these plants in the IEB test. Maize plants that had

not been in contact with aphids, were used as negative controls. Purified MDMV-ST and virus-infected source plants were positive controls. For electrophoresis, plants were either processed using the MDMV purification procedure (see Section III.C.2) or crude extracts were isolated. This latter procedure was done by grinding an equal weight of leaves in one volume of extraction buffer (III.A.1.c), removing plant debris by LS centrifugation and concentrating the extract by HS centrifugation. Samples for electrophoresis were disrupted in dissociation buffer (III.A.6.a.ix) at 1:1 ratio. 30-40 $\mu$ l of sample were applied to the gels. For IEB assay procedure, see III.G.2.

#### 1. RHOPALOSIPHUM PADI

One out of four maize plants had developed mosaic symptoms after aphid inoculation with MDMV-ST. Symptoms developed 9 days after inoculation. A maize plant grown from apical meristem tissue had not been successfully infected. In an IEB test with anti-MDMV-Win-1 IgG (1/30 dilution), extracts of plants showing mosaic symptoms gave positive results whereas extracts of plants with no symptoms were negative.

R. padi also transmitted MDMV-ST in a second transfer, from plants with mosaic symptoms, caused by aphid

inoculation. Five out of six plants developed mosaic symptoms and leaf tips became necrotic. Extracts of the plants with mosaic reacted positively with anti-MDMV-Win-1 and anti-MDMV-Kru-1 IgG in IEB tests. The discrepancy between the two experiments could be explained by the lack of skill in handling aphids in the earlier test.

## 2. DIURAPHIS NOXIA

In similar tests as the above, D. noxia transmitted MDMV-ST to fast germinating maize plants in 3/5 separate experiments (Table 29).

Table 29 : The number of plants infected with MDMV-ST after inoculation by Diuraphis noxia in aphid transmission tests.

| <u>Expt.</u> | <u>Number of plants with mosaic symptoms</u><br><u>Number of plants in test</u> |
|--------------|---------------------------------------------------------------------------------|
| 1            | 0/8                                                                             |
| 2            | 0/8                                                                             |
| 3            | 5/13 <sup>1</sup>                                                               |
| 4            | 3/23                                                                            |
| 5            | 2/11 <sup>2</sup>                                                               |

1=Three of these plants were used as source plants for a further transmission to fast germinating maize. Of these, 8 out of 24 developed mosaic symptoms.

2=The source plant in the experiment was a maize plant that had developed spontaneous mosaic symptoms.

Fig. 36 shows the results with extracts tested for MDMV in IEB test with anti-MDMV-Win-1 IgG (1/30 dilution). Strong positive reactions were observed at the 39Kd position in extracts from plants with mosaic symptoms (Fig. 36 lanes 2 and 5) but weak reactions were observed in plants with no symptoms (Fig. 36 lanes 3 and 4). Partially purified MDMV-ST and MDMV-Win samples are shown in Fig. 36 lanes 7 and 9 respectively and the extract of the source plant is shown in lane 1. Negative control was an extract of fast germinating maize not in contact with aphids (this lane was contaminated by flow-over of sample from lane 7).

From the above results, it can be concluded that both R. padi and D. noxia transmitted MDMV-ST.

These aphid experiments were done with groups of aphids and plants. Accurate single aphid transmission tests were not done. Transmission of MDMV by D. noxia has not been reported before.

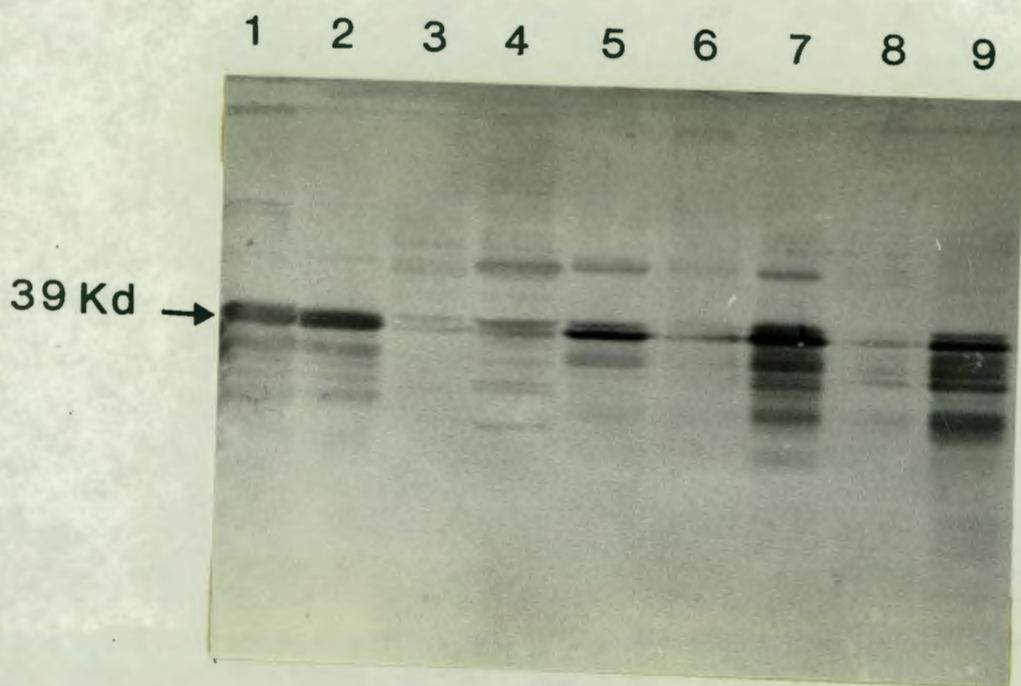


Figure 36 : IEB assay of extracts of maize plants to show that Diuraphis noxia transmitted MDMV-ST. The assay was done with 1/30 dilution anti-MDMV-Win-1 IgG.

- Lane 1 = MDMV-ST infected source plant
- 2 = Plant No.1 with mosaic symptoms after aphid inoculation
- 3 = Plant No.2 with no symptoms after aphid inoculation
- 4 = Plant No.3 with no symptoms after aphid inoculation
- 5 = Plant No.4 with mosaic symptoms after aphid inoculation
- 6 = Control fast germinating maize
- 7 = Partially purified MDMV-ST
- 8 = Molecular weight markers (Pharmacia)
- 9 = Partially purified MDMV-Win

#### E. ISOMETRIC VIRUSES FOUND IN MAIZE EXTRACTS

During the course of this investigation, it was found that spherical particles were sometimes isolated with the MDMV isolates. Electron micrographs of a differentially ultracentrifuged preparation of MDMV-Win illustrates the filamentous particles of MDMV-Win in Fig. 37 in association with spherical particles. The spherical particles appeared to be degraded, see Fig. 37.

In another experiment, clarified sap of MDMV-Win infected maize was reacted with anti-MDMV-Win-1 IgG to determine the antibody titre in the micro-precipitin test. (Sap was clarified with chloroform). The antigen/antibody drops were examined in a stereo microscope (Zeiss). A few of the dilutions were also observed in the electron microscope. Antibody-coated filaments were observed. Figure 38 shows the reaction with 1/256 dilutions of both antibody and antigen. There were also a large number of spherical particles with a diameter of  $\pm 27,7 - 33,3\text{nm}$ .

An MDMV-Kru preparation also showed spherical particles; MDMV-Kru was partially purified and examined by IEM assay using anti-MDMV-Win-1 antiserum as a probe. Only spherical particles, trapped by the antiserum, were observed. These were approximately 33nm in diameter, Fig. 39.

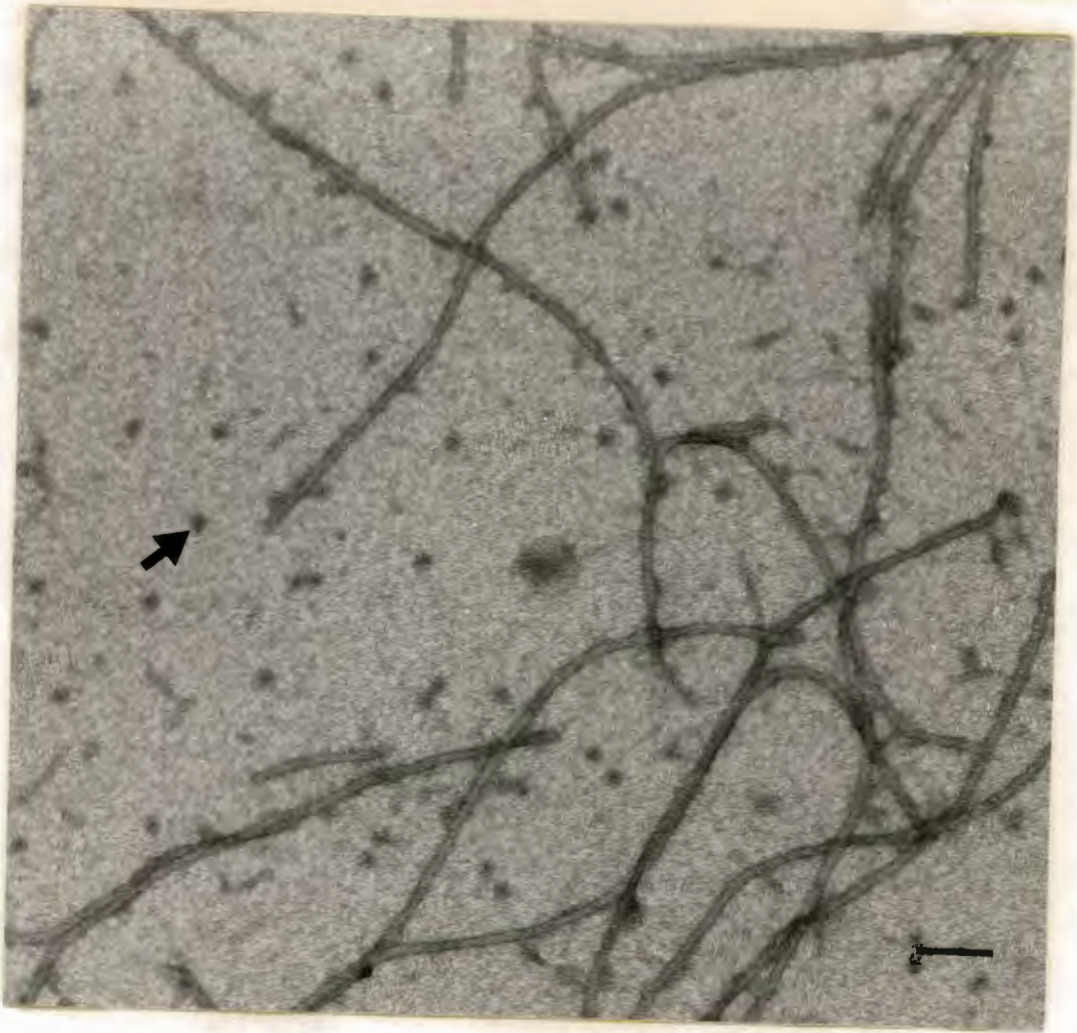


Figure 37 : Electron micrograph illustrating two different types of particle in a partially purified preparation of MDMV-Win:

- 1 - the filamentous MDMV
- 2 - the spherical particles that are partially degraded (indicated by the arrow).

The stain used was 2% uranyl acetate pH 6.0.

Magnification is 90 000 x. Bar represents 100nm.

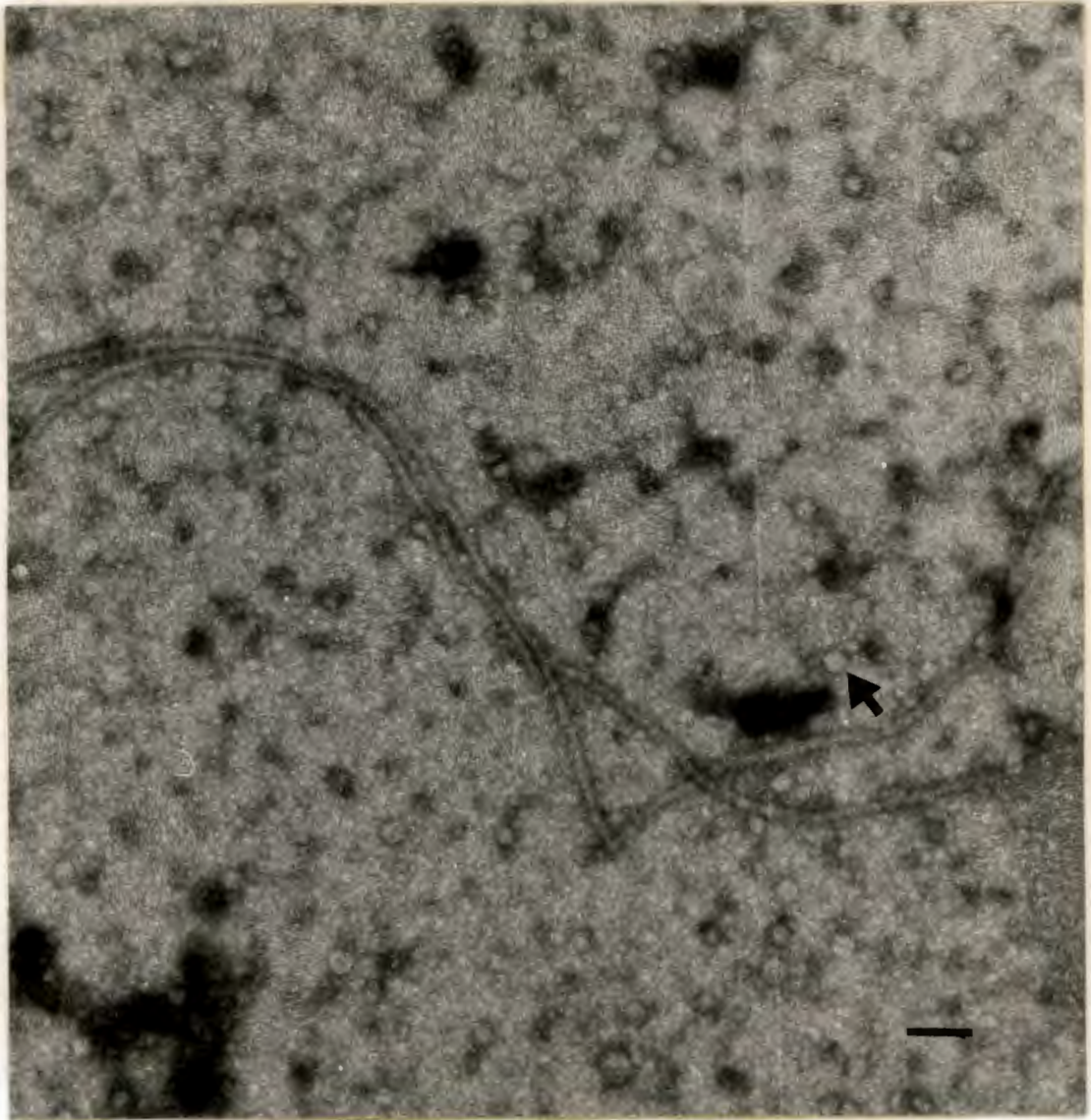


Figure 38 : Electron micrograph of precipitate of MDMV-Win Ag (1/256 dilution and anti-MDMV-Win-1 IgG (1/256 dilution) formed in the microprecipitin test. MDMV-Win Ag was chloroform clarified sap of infected maize. The micrograph shows the Ab-coated MDMV filaments as well as spherical particles (see arrow). The stain was 2% uranyl acetate pH 6,0. Magnification 90 000 x. Bar represents 100nm.

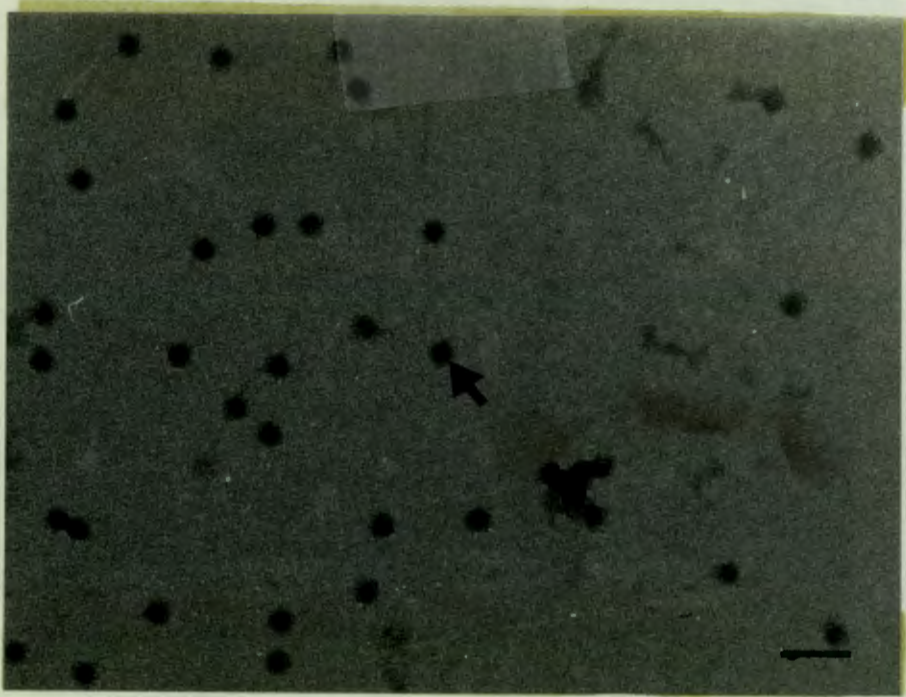


Figure 39 : ISEM of MDMV-Kru probed with anti-MDMV-Win-1 IgG. Only spherical particles (see arrow) were observed in this preparation. Magnification 90 000 x. The bar represents 100nm.

Larger and more regular spherical particles were also observed in MDMV-ST preparations (see Fig. 24).

Further, the preparation of concentrated fast germinating maize in which few filaments were observed, Fig. 32, also contained degraded particles which (with a little imagination) resembled degraded spherical particles.

From the above observations, it was found that spherical particles occurred in MDMV diseased maize plants. The identity of these particles is not resolved. Miss Elizabeth Knox (1983) showed that host absorbed antiserum to CMV reacted positively with extracts of maize plants. Prof. M.B. von Wechmar has observed (personal comm.) that CMV occurs frequently in maize and that inoculation of CMV strains onto maize produces dwarfed plants without any distinct symptoms and that CMV was recovered from these plants in quantity.

Looking at the present situation, it would appear that the CMV-like particles could have been destroyed by the purification procedure used to isolate MDMV. To avoid this, fast and slow germinating maize plants were processed by the method of Mossop et al (1976) for the isolation of CMV. The preparations were serologically tested for CMV. Slow germinating maize had been separated into two sets, one, with plants that had pale yellow symptoms and two, with no symptoms.

The isolation procedure was briefly as follows:

Leaves were extracted in 3 volumes (w/v) of  $0,1\text{MNa}_2\text{HPO}_4$  containing 1% thioglycollic acid and 1% Na di-ethyl dithio carbamate, pH8,0 in a Waring Blender.



Strained through cheesecloth



Centrifuged at LS 8 000g/10 minutes



Triton-X-100 added to supernatant to a final concentration of 2% (v/v) and stirred for 15 minutes at 4°C.



Centrifuged at 78 000g/2hr.



Resuspended pellets in 1/10 the original volume of extraction buffer.



Centrifuged at LS 5 500g/15 minutes.

The samples were dissociated with an equal volume of disruption buffer, and were electrophoresed on 12,5% polyacrylamide gels and reacted with anti-CMV, anti-MDMV-Kru-1 and anti-MDMV-Win-1 antibodies in the IEB assay. CMV antigen and host absorbed antisera were kindly donated by P. Lupuwana. The slow germinating and fast germinating samples did not react at the major MW position for the MDMV-ST protein when probed with anti-MDMV-Win-1 IgG,

Fig. 40. There was a weak reaction at the 38Kd position in lanes 4 and 5 containing the slow germinating samples. The reaction with host absorbed anti-MDMV-Kru-1 antiserum was similar. CMV antigen did not react with anti-Win-1 IgG, Fig. 40, lane 1.

A reciprocal test was done with anti-CMV antisera but due to the presence of a number of host bands, clear cut results for the presence of CMV in the fast and slow germinating samples could not be determined.

To determine whether MDMV antisera recognised CMV antigen (i.e. contained antibodies directed against CMV), ELISA tests were done using three different strains of CMV, which were kindly donated by Mr. P. Lupwana.

In the first test a CMV strain obtained from tobacco by P. Lupwana was tested against anti-MDMV-Win-1 IgG and the homologous CMV IgG in the sandwich ELISA test. Both the coating antibody and the specific alkaline conjugated IgG were diluted at 1/400. Anti-MDMV-Win-1 IgG did not recognize the CMV strain. In the reciprocal test, CMV serum did not recognize the MDMV antigen, Table 30.

Table 30 : Sandwich ELISA to determine whether anti-MDMV-Win-1 IgG recognized CMV antigen

| Antibody          | Absorbance at 405nm      |                     |                    |                            |                      |                                 |  |
|-------------------|--------------------------|---------------------|--------------------|----------------------------|----------------------|---------------------------------|--|
|                   | CMV infected tobacco sap | Healthy tobacco sap | MDMV-Win 0,12mg/ml | MDMV-ST infected maize sap | Uninfected maize sap | Concentrated maize <sub>1</sub> |  |
| <u>Test No. 1</u> |                          |                     |                    |                            |                      |                                 |  |
| Anti-MDMV-Win-1   | 0,012                    | 0,006               | 2,4                | - <sup>2</sup>             | 0,070                | 0,003                           |  |
| Anti-CMV-1        | 2,9                      | 0,007               | 0,002              | -                          | 0,000                | 0,000                           |  |
| <u>Test No. 2</u> |                          |                     |                    |                            |                      |                                 |  |
| Anti-MDMV-Win-1   | -                        | -                   | 2,9                | 2,9                        | 0,133                | -                               |  |
| Anti-CMV-1        | 0,508                    | 0,033               | -                  | 0,045                      | 0,031                | -                               |  |

1=Concentrated preparation of uninoculated maize grown from tissue culture.

2=Not done.

In another experiment, two further strains of CMV were tested against anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-MDMV-ST IgG in the sandwich ELISA test. The purified CMV strains, CMV-impatiens (isolated from Impatiens, Pretoria) and CMV-lupine (isolated from infected lupines from Western Cape) were kindly donated by P. Lupuwana. These antigens were diluted 5-fold starting with a concentration of 2mg/ml. The positive control was MDMV-ST infected maize sap and the negative control was uninoculated maize sap. Anti-MDMV-Kru-1 and anti-MDMV-ST IgG did not detect either CMV strain, but anti-MDMV-Win-1 IgG recognized only CMV-lupine and not CMV-impatiens, Table 31.

These results showed that anti-MDMV-Win-1 contains a low level of antibodies that recognised CMV-lupine. Prof. M. B. von Wechmar (pers. comm.) has shown that CMV-lupine infects maize and that this virus can be purified from maize.

Table 31 : Sandwich ELISA to test whether three MDMV antisera contained antibodies to two CMV strains.

| <u>Antibody</u> | <u>Antigen</u>             | <u>Absorbance at 405nm</u> |                       |                       |                       |                       |
|-----------------|----------------------------|----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                 |                            | <u>5<sup>0</sup></u>       | <u>5<sup>-1</sup></u> | <u>5<sup>-2</sup></u> | <u>5<sup>-3</sup></u> | <u>5<sup>-4</sup></u> |
| Anti-MDMV-Win-1 | CMV-lupine                 | 0,468                      | 0,142                 | 0,051                 | 0,020                 | 0,012                 |
|                 | CMV-impatiens              | 0,014                      | 0,015                 | 0,016                 | 0,010                 | 0,015                 |
|                 | MDMV-ST infected maize sap | 2,18                       | 2,18                  | 1,632                 | 0,811                 | 0,235                 |
| Anti-MDMV-Kru-1 | Uninfected maize sap       | 0,102                      | 0,100                 | 0,133                 | 0,076                 | 0,015                 |
|                 | CMV-lupine                 | 0,114                      | 0,056                 | 0,033                 | 0,024                 | 0,019                 |
|                 | CMV-impatiens              | 0,024                      | 0,033                 | 0,023                 | 0,025                 | 0,024                 |
|                 | MDMV-ST infected maize sap | 2,088                      | 1,824                 | 1,376                 | 0,827                 | 0,324                 |
|                 | Uninfected maize sap       | 0,034                      | 0,033                 | 0,033                 | 0,030                 | 0,025                 |
| Anti-MDMV-ST    | CMV-lupine                 | 0,151                      | 0,140                 | 0,084                 | 0,083                 | 0,086                 |
|                 | CMV-impatiens              | 0,104                      | 0,071                 | 0,076                 | 0,076                 | 0,087                 |
|                 | MDMV-ST infected maize sap | 0,983                      | 0,871                 | 0,515                 | 0,340                 | 0,168                 |
|                 | Uninfected maize sap       | 0,274                      | 0,356                 | 0,320                 | 0,193                 | 0,122                 |

F. TESTING OF FIELD COLLECTED MAIZE FOR MDMV

RHOODEPLAAT ISOLATES

During a visit to the Rhoodeplaar Horticultural Research Institute in January, 1984, infected maize plants were collected from outdoor trials.

Three different symptoms were observed:

Isolate A - yellow green mosaic symptoms

Isolate B - yellow green mosaic symptoms and parallel yellow stripes

Isolate C - crinkled leaves

Leaves of these plants were examined for the presence of MDMV in the ELISA test.

Crude extracts were prepared as follows. Leaf samples were ground 1:3 (w/v) in  $KPO_4$  pH7,0 buffer and filtered through cheesecloth. The samples were diluted 4-fold and tested against MDMV-antisera (1/300 dilutions) in the sandwich ELISA test. Control samples of uninfected fast germinating maize and MDMV-ST infected maize were tested similarly.

Rhooideplaat A isolate reacted strongly with anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-SCMV 4975-1 antibodies. The results are shown in Table 32. Rhooideplaat B and C isolates reacted negatively with both anti-MDMV-Kru-1 and anti-SCMV 4975-1 antibodies and showed very weak reactions with anti-MDMV-Win-1 antibody.

Rhooideplaat A, B and C extracts, from original field collected material, each showed the presence of filamentous virus-like particles when examined as leaf crush samples in the electron microscope. Rhooideplaat A and B were inoculated onto maize seedlings. After one week the maize showing mosaic symptoms, was examined. The leaf crush samples of Rhooideplaat A and B again showed filamentous particles.

These tests confirm that MDMV was present in Sample A. As the ELISA test was negative for isolates B and C, but the electron microscope examination for B and C also showed filamentous particles, it is possible these may have been of the MDMV-A type. At the time of examination, this could not be followed up as anti-MDMV-A serum was unavailable.

The initial symptoms of the field-infected samples were not typical for that of an MDMV-infection only. Professor von Wechmar initiated a further examination of the B sample

and showed that the plant was also infected with another virus reacting positively with anti-CMV-serum. Isolate B also infected squash, cucumber and tobacco plants. (Von Wechmar, pers. comm.)

Table 32 : The results of the maize isolates, Rhooideplaat A, B and C tested with anti-MDMV and anti-SCMV antibodies in the sandwich ELISA.

| <u>Antigen</u>                   | <u>Absorbance at 405nm<sup>1</sup></u> |                        |                         |
|----------------------------------|----------------------------------------|------------------------|-------------------------|
|                                  | <u>Anti-MDMV-Win-1</u>                 | <u>Anti-MDMV-Kru-1</u> | <u>Anti-SCMV 4975-1</u> |
| Rhooideplaat A                   | 2,8 ± 0                                | 2,5 ± 0                | 2,9 ± 0                 |
| Rhooideplaat B                   | 0,284 ± 0,013                          | 0,080 ± 0,004          | 0,088 ± 0               |
| Rhooideplaat C                   | 0,620 ± 0,029                          | 0,058 ± 0,004          | 0,064 ± 0,003           |
| MDMV-ST                          | 2,8 ± 0                                | 1,866 ± 0,032          | 2,8 ± 0                 |
| Fast germinating maize (Control) | 0,426 ± 0,019                          | 0,082 ± 0,018          | 0,048 ± 0,003           |

1=Average of 2 readings.

CHAPTER V  
DISCUSSION

In the present study the South African isolates from maize were identified as maize dwarf mosaic virus strain B (MDMV-B) as they were all found to be serologically related to the MDMV-B strain obtained from Dr. D. Gordon (Ohio State University). All three isolates were shown to be distinct from the MDMV-A strain obtained from the same source.

As the viruses under study were isolated from maize it was decided to follow the U.S. nomenclature system and to call them MDMV and not SCMV as was done previously by Professor von Wechmar. Earlier references to South African isolates by von Wechmar (1967, 1983, von Wechmar and Hahn, 1967) identified the virus as sugarcane mosaic virus as she could not show significant differences between the maize and sugarcane isolates tested at the time.

The literature abounds with a confusion in nomenclature of the SCMV- and MDMV- strains, most of which came about due to the inaccurate biological criteria used in the different studies, i.e. symptom expression, differential hosts and aphid transmission (Ford and Tosic, 1972; Abbot and Tippet, 1966; Louie and Knoke, 1975).

Various other reasons exist for this confusion, some due to

historical developments and others which are discussed in some detail in recent papers by Gordon (1984) and Gillaspie et al. (1984). Some of these are: a) naming of the strains by assigning letters, i.e. MDMV-A, b) naming of the strains after the hosts in which they are found, i.e. Johnsongrass strain of SCMV, or , c) naming the strain after the symptom produced, i.e. sorghum red stripe.

A uniform set of criteria is needed for accurate strain differentiation. Gillaspie et al. (1984) suggests a uniform set of differentials to be tested for host reactions under standard conditions. As this is very difficult to achieve and will not show up masked or latent infections easily, more stringent testing procedures should be used to re-investigate the existing differences reported for isolates and strains described in various laboratories. Preferably these should include sensitive immunological, biochemical and biophysical testing procedures (Tosic and Ford, 1974; Sum et al., 1979; Langenberg, 1974; Hill et al., 1973; Moghal and Francki, 1976, 1981; Gough and Shukla, 1981; Shukla and Gough, 1984; Berger and Toler, 1983a; von Wechmar and Chauhan, 1984).

The present study has shown that several maize cultivars that were examined contained seed transmitted MDMV-B. Some of the plants grown from such seed developed symptoms but others appeared to contain the virus without producing symptoms (see section IV.C).

The presence of seed transmitted MDMV (MDMV-ST, see section IV.C) in maize complicates the study of strains of this virus. The present study showed that the seed transmitted virus is a virulent isolate and it is quite possible that during the early part of this study - when we were unaware of the presence of the symptomless seed transmitted MDMV in the maize - the MDMV isolates were propagated and became contaminated with this seed transmitted MDMV. This could be a possible reason for not finding significant differences amongst the local isolates. It is, of course, possible that all three isolates originated as seed transmitted virus in the first place in field grown maize and may have had a common origin.

The MDMV-ST was shown to be indistinguishable from the three other South African isolates and the U.S. MDMV-B strain when tested immunologically in ELISA, IEB and ISEM by using antisera to the isolates and the MDMV-B strain. The reciprocal test with MDMV-B (U.S.) antigen with our anti-MDMV serum was not possible as the U.S. MDMV-B virus was not propagated in South Africa.

During the early part of this project it was desirable to have a known standard virus for purposes of comparison. Gillaspie's SCMV 4975 (U.S.A) was obtained and propagated for this purpose. Initial relationship studies between the local isolates and SCMV 4975 showed detectable differences

(section IV.6 and 9) (von Wechmar, 1983). These differences showed up in the IEB assay when anti-MDMV-Kru and anti-MDMV-Bar were used to probe proteins of SCMV 4975, MDMV-Kru, -Win and -Bar blotted onto nitro-cellulose sheets. The antisera used for this test were all early bleeds. When this test was later repeated with later antiserum to MDMV-Kru, SCMV 4975 protein was recognised strongly, and differences detected earlier were no longer recognised (see Fig. 14d).

It is unlikely that the early bleeds of antisera used in the first test were solely responsible for the observed differences in heterologous titres and in the very weak reaction observed in the blots with SCMV 4975. A more likely explanation for this discrepancy is a possible contamination of the SCMV 4975 isolate by seed transmitted MDMV<sup>\*</sup> which was only observed and investigated much later. The validity of the observed difference between Gillaspie's SCMV 4975 and the South African isolates will have to be re-examined once virus-free maize is available for virus propagation.

The availability of sensitive immunological techniques certainly helped to detect low levels of seed transmitted virus as shown in ELISA tests (see Table 26). The availability of high titred antisera used in ELISA tests, proves to be a superior way to test the percentage of seed transmitted virus in seed sources compared to conventional sap-

\* (when propagated in K.E.P. Maize)

transmission and symptom evaluation. Low incidences of seed transmission of MDMV-A have been reported, e.g. Tosic and Susic (1977) tested 12 000 seeds and reported seed transmission in 0,008% seed, while Panayatou (1981) tested 12 500 seedlings but no seed transmitted virus was detected when he examined them for mosaic symptoms. The concentration of virus calculated from values obtained in ELISA tests in this study varied from 7 to 125 ug/ml per coleoptile.

A detailed analysis of the distribution of MDMV in different seed lots was not made, but a visual determination for seed transmission was done in approximately 800 seedlings of which 1,2% seedlings showed mosaic symptoms. From visual examination and ELISA tests, it appeared that the fast germinating seeds did not contain MDMV. The rate of germination was an indication of the absence or presence of MDMV. No MDMV was found in the fast germinating seeds of cv. K.E.P.-Witplat. MDMV was found in high proportion (6/11) of slow germinating seeds (see section IV.C.4). This criterion proved valuable in further work where fast germinating seedlings were used to propagate MDMV isolates in order to avoid further possible contamination.

For successful propagation of MDMV-A it would appear to be important to avoid contamination of MDMV-B by indiscriminate propagation in unselected maize seedlings. At the time of testing, the MDMV-A isolate had not been propagated in maize.

It may be a good practice to avoid serial propagation in maize to keep the strain pure. Virus-free maize or possibly alternate hosts such as Johnsongrass or sorghum may be used where virus is required for antiserum production and characterization of strains. Gillaspie (1972) and Pirone (1966) found that sorghum was a convenient host from which to purify SCMV whereas Tomic et al (1974) reported that a large amount of pigment was produced when purifying SCMV or MDMV from sorghum leaves.

The Johnsongrass-isolate-1, which may be any one of the MDMV-A group of strains (-A, -C, -D, -E or -F) was only found towards the end of the project, and unfortunately antiserum to this strain of MDMV was not yet available for doing the necessary comparative tests (see section IV.B.2). The tests done with MDMV-B related antisera, showed no reaction in the ISEM but a weak reaction in IEB tests. This reaction may be that of cryptotopes which would not be recognised as such in electron microscopy tests - a test which relies on intact virions. Rybicki and von Wechmar (1982) have shown that distantly related strains of TMV could be detected in IEB tests. Therefore it is not surprising that MDMV-B specific antisera (anti-MDMV-Win-1, anti-MDMV-Kru-1 and anti-MDMV-ST) would recognise the Johnsongrass-isolate-1 in IEB tests. Also MDMV-A and -B are related but serologically different as shown in microprecipitin tests by MacKenzie et al (1966), Tomic and Ford (1974) and more recently by Jarjees and

Uyemoto (1984) in ELISA tests.

Due to the absence of antisera to a South African isolate of MDMV-A, it was not possible to test for the presence of MDMV-A at the beginning of the project. At this point we therefore have no knowledge whether MDMV-A occurs in South African maize crops or only in Johnsongrass or possibly in in some other grasses (not yet tested). This aspect needs further investigation as Johnsongrass is a perennial overwintering grass in the Transvaal maize producing areas, which is also the case in North America.

From this it can be concluded that the source of MDMV-B in maize fields (i.e. MDMV-Win, -Kru, -Bar) could not be due to spread from Johnsongrass plants, as the virus obtained from the latter is a different strain. The source of MDMV-B in the field is not known but a strong possibility is the spread by seed contamination. The extent of this must still be determined.

The filamentous particles found in Bromus (Abrahamskraal isolate) and the barley isolates were not identified as MDMV, although weak reactions were obtained with MDMV-B antisera in IEB tests. Tests with anti-WSMV-serum indicated a possible relationship of Abrahamskraal isolate to WSMV. The barley-isolate-1 and Abrahamskraal also share common serological properties as indicated in IEB tests with Abrahamskraal-antiserum. But further serological tests are required for

more exact identification of these isolates.

The spherical particles found in maize were not positively identified but tests with CMV antisera and antigen, indicated that they may be CMV. CMV has been reported to occur naturally in maize (Boothroyd, 1981) and aphid-borne infections of CMV on maize have been reported, although CMV is not considered a major disease of maize (Gordon et al, 1981).

Although MDMV-ST preparations, when tested with anti-CMV antiserum in IEB tests, were positive, CMV was not successfully purified from maize, using the method of Mossop et al, 1976.

Only degraded spherical particles were observed in MDMV preparations. CMV is known to be an unstable virus and the MDMV purification procedure, with chloroform clarification and concentration with PEG 6000, caused dissociation of CMV. Particles of CMV also become disrupted when mounted unfixed in neutral PTA but are stable in uranyl acetate pH 4,5 (Francki et al, 1979; Francki and Hatta, 1980).

When von Wechmar showed that seed-transmitted BMV occurred in small grain seed (von Wechmar et al, 1984) and that seed-transmitted MDMV could be detected in maize (von Wechmar and Chauhan, 1984) and seed-borne CMV-like viruses were detected in germinated maize seed (E. Knox, Hons. project, 1983), it

appeared important to examine whether seed-borne icosahedral viruses were also present in maize used in this study. Due to the great instability of the CMV particle (it dissociates very rapidly during isolation), it was not detected as a contaminating virus particle in purified virus preparations. Occasionally spherical particles had been observed (Fig. 37) but this was inconsistent and could therefore not be explained as it was difficult to follow up. This does not exclude the possibility that CMV-like protein subunits could have been present in the MDMV preparations that were injected for antiserum production, and that the immune system of the rabbit could then have amplified this by producing antibodies to both the MDMV and to the dissociated CMV-protein.

For this reason it was felt necessary to examine some of the MDMV antisera for the presence of antibodies that would recognise CMV. At the time when this work was done, the effect of CMV-infection on maize was examined by Professor von Wechmar. It was found that maize plants inoculated with certain isolates of CMV showed no symptoms, except being dwarfed compared to uninoculated plants. However, measurable quantities of virus could be isolated from these plants (pers. comm. von Wechmar). Knowing this, it is possible that CMV infection can easily go undetected unless plants are specifically tested for it by a sensitive immunological assay. Whether the dead growth points, as illustrated in Fig. 2, are due to the presence of seed-borne CMV is

uncertain and still needs to be examined. Alternatively, this abnormal reaction in slow germinating maize could also be the effect of a dual infection of MDMV and CMV, thus resulting in a synergistic effect. This aspect is currently being investigated as part of another project.

When the MDMV antisera were tested in ELISA (see Table 31), it was found that anti-MDMV-Win-1 and anti-MDMV-Kru-1 reacted with an isolate of CMV (CMV-lupine). The cross reactivity of the CMV antisera with the MDMV antigen is not yet understood (see Figs. 27 and 28).

The implications of finding seed-transmitted MDMV and possibly CMV, is that maize crops may be infected in the field with both MDMV and CMV.

As both these viruses are transmitted by aphids, the viruses may be spread rapidly through crops in the presence of aphids. Even if symptoms are not severe, the presence of one or two viruses may make the plants more vulnerable to adverse weather conditions such as sudden hot dry winds and drought.

### CONCLUSION

MDMV strain B was found in naturally infected maize in the Transvaal and Natal, South Africa. Another strain isolated from Johnsongrass was shown to have only a distant relationship to the MDMV-B isolates and in all probability is an MDMV-A strain. It differs sufficiently from MDMV-B to be called a different strain. Confirmatory tests to ascertain whether it is identical to the U.S. MDMV-A strain are still outstanding.

Filamentous viruses isolated from barley and bromegrass were shown not to be related to MDMV-B. Positive results with WSMV antiserum seem to indicate that the Abrahamskraal-isolate was related to WSMV and not to MDMV-B. No relationship of the Barley-isolate-1 and -2 to MDMV-B was detected. At this stage their identity remains unknown.

SCMV isolated from South African grown sugarcane appeared to differ very little (if at all) from MDMV-B isolated maize.

Seed transmitted MDMV-B was found in two old commercial cultivars as well as in the two contemporary commercial hybrids. A detailed analysis of the extent of seed-borne MDMV-B in all the different commercial hybrids has not been made.

For crop safety it is certainly necessary that the level and incidence of seed-borne MDMV in commercial hybrid seed is known. Similarly, the virus content of germplasm used in national breeding programmes should be investigated to ensure that future progeny maize hybrids are virus-free.

The epidemiology of MDMV-B has not been looked at. It is unknown whether overwintering hosts from which new seasonal infections would arise by way of aphid transmission, exist in South Africa. Seed-borne MDMV-B may certainly play an important role in the geographical (vertical) dissemination of this virus and be the cause of primary infection loci. Secondary aphid transmission (in seasons/periods favourable to aphids) may then be responsible for horizontal spread.

Diuraphis noxia was shown to transmit the seed-borne MDMV-B isolate from maize to maize. This is the first report of MDMV-B transmission by D. noxia, although other aphid species are known to be effective vectors. Under laboratory conditions D. noxia colonised maize plants. This has not yet been observed in nature and only time will show whether this new invader aphid will colonise maize naturally and thus become an important natural vector as has happened with BMV in wheat.

Several incidences of natural MDMV-B infected maize fields were examined by Professor von Wechmar. Where present, the disease had a severe effect on the plants. Yield-loss determinations were not made except in one incidence where a crop loss was recorded and could be attributed to a severe infection from MDMV-B during the early growth period (von Wechmar, pers. comm.). Seed-borne MDMV-B was present in an aliquot of the seed sown.

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