

The occurrence of maize mosaic virus on sorghum in India¹

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Summary

A leaf disease of sorghum (*Sorghum bicolor*) characterised by fine discontinuous chlorotic streaks between the veins, was observed on sorghum grown during the 1987/88 post-rainy season in peninsular India. Early-infected plants were stunted, had shortened internodes, and produced poorly developed panicles. The virus was transmitted by the delphacid planthopper, *Peregrinus maidis*. Negatively stained leaf dip preparations contained bullet-shaped virus particles ($208 \pm 4.4 \times 66 \pm 1.0$ nm) resembling those of rhabdoviruses. In ultrathin sections, the particles budded through the inner nuclear membrane and were present in the cytoplasm within membrane-bound vesicles that were apparently contiguous with the distended outer nuclear membrane. A method for purifying the virus was developed utilising polyethylene glycol (PEG) precipitation, Celite filtration and sucrose density-gradient centrifugation. An antiserum was produced in rabbits with a titre of 1/2650 in the precipitin ring interphase test. The virus could be detected in infected sorghum leaf tissues using a direct antigen coating form of enzyme-linked immunosorbent assay (DAC-ELISA). In immuno-double diffusion tests, the virus reacted positively with antisera to maize mosaic virus (MMV) from Reunion (MMV-RN) and Hawaii (MMV-HI), but not with antisera to barley yellow striate mosaic (BYSMV), cereal chlorotic mottle (CCMV), and cynodon chlorotic streak (CCSV) viruses. Thus, the virus isolated from sorghum is designated the MMV-S isolate. In DAC-ELISA tests, MMV-S reacted positively with antisera to MMV-R, MMV-HI, MMV-Florida isolate, CCSV, and CCMV, and weakly with antiserum to BYSMV. SDS-polyacrylamide gel electrophoresis revealed four major proteins of relative mass M_r 70 000, 59 000, 32 000 and 28 000. In electro-blot immunoassay, MMV and CCSV antisera detected the G and N proteins. These data suggest that MMV-S should be placed in the sonchus yellow net virus subgroup of plant rhabdoviruses.

Introduction

Diseases characterised by discontinuous chlorotic streaks between the veins, stunting and severe yield reductions have been reported on sorghum (*Sorghum bicolor* (L.) Moench) in India by several workers (Capoor, Rao & Varma, 1968; Cherian & Kylasam, 1937; Herold, 1972; Mali & Bhagwat, 1975). The causal agents of these diseases were not identified. Our surveys in the post-rainy season of 1987/88 (November–April) revealed that a similar disease was widely distributed in sorghum crops, causing severe yield losses in peninsular India. Rhabdovirus-like particles were observed in negatively stained leaf-dip preparations from infected leaves. The delphacid planthopper, *Peregrinus maidis* (Ashm.), occurred abundantly

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in affected fields and transmitted the rhabdovirus associated with the disease. We report here the transmission, purification, serological relationships, and protein analysis of this rhabdovirus.

Materials and Methods

Virus source and culture. The original virus source was a naturally-infected sorghum plant in research plots at the ICRISAT Center. Following identification of the planthopper vector, a healthy colony of *Peregrinus maidis* was reared on sorghum in insect-proof wire-mesh cages in a glasshouse. First or second instar nymphs were collected and released onto 6 wk-old healthy sorghum plants. The nymphs derived from first-generation adults were collected and released onto another batch of 6 wk-old healthy sorghum plants. Serial transfers of planthoppers were made for five generations, and all the planthopper-fed sorghum plants were checked for the absence of virus to make sure that the colony was virus-free. Virus transmission with *P. maidis* was done as described by Gingery, Nault, Tsai & Lastra (1979), Tsai (1975), and Tsai & Zitter (1982). Nymphs of the last-instar stage were allowed a 3-4 days acquisition access period on infected sorghum plants, and after a 2 wk incubation period on healthy sorghum plants, they were transferred to 3 - 4 wk-old healthy sorghum seedlings to produce a continuous supply of infected plants in a glasshouse.

Electron microscopy. Leaf pieces of infected sorghum were prefixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and then washed thrice in the buffer, allowing 30 min for each wash. The pieces were postfixed in 2% aqueous osmium tetroxide for 6 h, followed by three washings in distilled water. Tissues were dehydrated in a graded acetone series, followed by embedding in Spurr's medium (Spurr, 1969). Sections were stained in uranyl acetate and lead citrate, and examined in a Philips 201C electron microscope. Leaf dip and purified preparations placed on carbon-coated grids were fixed for 10 min by inverting grids on a drop of buffered 1% glutaraldehyde. The grids were then washed with water and stained with 1% aqueous uranyl acetate, pH 4.0.

Virus purification. All operations were carried out at or near 4°C. Fresh, infected sorghum leaves were triturated in a blender in an extractant containing 0.1 M glycine-NaOH, 0.01 M MgCl₂ (0.1 M Gly-Mg), pH 8.0 and 0.04 M Na₂SO₃ (1:4, w/v). The homogenate was filtered through two folds of muslin cloth and centrifuged at 5000 rpm (Sorvall RC 5) for 10 min. The virus was precipitated by the addition of 0.1 M NaCl and 6% polyethylene glycol (w/v). After centrifugation at 10 000 rpm for 15 min, the resulting pellet was suspended in 0.1 M Gly-Mg, pH 7.2, and clarified at 5000 rpm for 10 min. The supernatant was subjected to rate-zonal density gradient centrifugation for 30 min at 23 000 rpm in a Beckman SW28 rotor, in sucrose gradients made of 7 ml each of 10, 20, 30 and 40% (w/v) in 0.1 M Gly-Mg buffer, pH 7.2. A light-scattering virus zone at a height of 2.5 to 3.0 cm was collected and filtered through Celite 545 (Johns-Manville; Shirako & Ehara, 1985). The milky-white filtrate was diluted in 0.1 M Gly-Mg buffer, pH 7.2, and subjected to two cycles of quasi-equilibrium zonal density gradient (made of 7 ml each of 30, 40, 50, and 60% w/v sucrose in 0.1 M Gly-Mg, pH 7.2) centrifugation for 2.5 h at 23 000 rpm in a SW 28 rotor. A light-scattering virus band at a height of 3.8 to 4.2 cm was collected, diluted in 0.1 M Gly-Mg, pH 7.2, and pelleted at 20 000 rpm for 1.5 h in a SW 28 rotor. The final pellet was suspended in 0.01 M phosphate buffer, pH 7.2.

Serology. New Zealand White inbred rabbits were given three intramuscular injections at weekly intervals, followed by a fourth injection 2 wk after the third. Purified virus suspension (1 ml), obtained from 50 g sorghum tissue, was emulsified with 1 ml of Freund's incomplete adjuvant and injected into the hind leg of the rabbit at three different sites. Bleeding

began 1 wk after the last injection. Serum was stored at -70°C .

Antiserum titres were determined by the precipitin ring interphase test as described by Reddy & Black (1966). Serological relationships were tested by the Ouchterlony gel double diffusion test in 0.85% (w/v) agarose in phosphate buffered saline (PBS; 0.02 M phosphate, pH 7.5, 0.15 M NaCl, 0.5% NaN_3). Crude sap antigens were prepared by grinding leaves with a mortar and pestle in PBS containing 1% Triton X-100 and squeezing the sap through cheesecloth. Test antigens were placed in the central well and the antisera in the peripheral wells. Plates were kept in a humid chamber at room temperature and observed daily for up to 1 wk. Antisera used in this study were generous gifts from others working with cereal rhabdoviruses; MMV Reunion isolate (MMV-RB), from Dr M. Peterschmitt, Institute of Research for Tropical Agriculture, Reunion Island; Hawaiian isolate (MMV-HI) from Dr D.T. Gordon, Ohio Agricultural Research and Development Center, Wooster, USA; Florida isolate (MMV-FL) from Dr B.W. Falk, University of California, Davis, USA; and cynodon chlorotic streak virus (CCSV), cereal chlorotic mottle virus (CCMV), and barley yellow striate mosaic virus (BYSMV) from Dr B.E.L. Lockhart, University of Minnesota, St Paul, USA.

Enzyme-linked immunosorbent assay. A direct antigen coating method (DAC-ELISA) was used, as described by Hobbs, Reddy, Rajeshwari & Reddy (1987). Antisera were cross-adsorbed with healthy sorghum leaf extracts, also as described by Hobbs *et al.* (1987). Rabbit Fc-specific immunoglobulins from antiserum produced in goats (Cappel Laboratories Inc., West Chester, PA) were conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) and used at 1:1000 dilution; p-nitrophenyl phosphate was used as a substrate. Absorbance readings ($A_{405\text{ nm}}$) were taken with a Dynatech Micro-ELISA Minireader MR 590 calibrated to zero without an ELISA plate on the reader. All the assays were duplicated and each experiment repeated thrice.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis of virus proteins was done according to Laemmli (1970). Samples were prepared by mixing, at 1:1 ratio, purified virus and two-fold concentrated sample buffer (0.0625 M Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 0.5% 2-mercaptoethanol, and 0.001% bromophenol blue), which were then boiled for 5 min before loading onto the gels. A 10% resolving gel prepared in 0.375 M Tris-HCl, pH 8.9, and 3.3% stacking gel prepared in 0.125 M Tris-HCl, pH 6.8, was used for analysing the proteins. Electrophoretic separation was at a constant 30V for 16 h at room temperature. Gels were stained with 0.2% Coomassie Brilliant Blue R250 (Bio-Rad) in 50% methanol, 7% acetic acid for 1 h, and destained in 20% methanol, 7% acetic acid overnight at room temperature. Phosphorylase B (92 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400) from Bio-Rad were used as the molecular weight standards.

Electro-blot immunoassay. Electrophoretic transfer of viral proteins was done as described by Burgermeister & Koenig (1984). After electrophoretic transfer of proteins, the nitrocellulose membrane (Schleicher & Schull BA85, 0.45 μm pore size) was incubated on a shaker overnight in a solution of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and then incubated for 1 h in a 1/5000 dilution of antiserum in TBS with 0.05% Tween-20 (TTBS) containing 1% BSA (TTBS-BSA). After washing three times for 5 min each in TTBS, the membrane was incubated for 1 h in a 1/500 dilution of alkaline phosphatase-labelled goat anti-rabbit Fc-specific antibodies in TTBS-BSA. The nitrocellulose membrane was washed three times for 5 min each in TTBS and transferred to a substrate solution containing 5 mg 1-naphthylphosphate in 20 ml 0.2 M Tris-HCl, pH 8.3. Colour development was initiated by the addition of 25 mg Fast Blue RR salt (Sigma) in 5 ml water. Colour development was recorded visually and stopped by washing the membrane in distilled water.

Results

Symptoms

Symptoms appeared on plants 2-3 wk after exposure to viruliferous planthoppers. Initial symptoms were chlorotic steaks between the veins (Plate 1, fig. 1), which sometimes became necrotic at an advanced stage of disease development. When infection was severe, the whole leaf was chlorotic. Infected plants were severely stunted when infected as 2-3 wk old sorghum seedlings; panicles produced by such plants contained very few seeds. Plants inoculated as 5-6 wk-old seedlings were not severely stunted; nevertheless, panicles produced were small and contained fewer grains than non-inoculated control plants.

Electron microscopy

Large, densely packed aggregates of rhabdovirus particles were observed in many cells. In some sections, the entire cytoplasm was filled with particles (Plate 1, fig. 2a). These particles apparently budded through the inner nuclear membrane, perpendicular to the membrane surface. Occasionally, virus particles were seen within an expanded region of the perinuclear space (Plate 1, fig. 2b), and the viral envelope appeared continuous with the inner nuclear membrane. No particles were observed within nuclei, chloroplasts or mitochondria, or individually in the cytoplasm. Virus particles observed in the cytoplasm were surrounded by a membrane forming large cisternae with many branches that appeared to be contiguous with the distended outer nuclear membrane. In cross section, virus particles showed a nucleocapsid, encircled by an envelope membrane with spikes (Plate 1, fig. 2c).

In leaf dip preparations fixed with 1% glutaraldehyde and stained with 1% uranyl acetate, the majority of the virus particles appeared bullet-shaped, but bacilliform particles were occasionally observed (Plate 2, fig. 3a). The bullet-shaped particles measured $208 \pm 4.4 \times 66 \pm 1.0$ nm. In unfixed preparations, the majority of the particles were pleomorphic. End-to-end aggregation of the particles was also observed occasionally (Plate 2, fig. 3b).

Virus purification

In initial experiments, tris buffer was compared with glycine buffer (Falk & Tsai, 1983) for virus purification. We consistently obtained more virus (as measured by electron microscopic counts of particles) with glycine buffer. Therefore, glycine buffer was used for extraction and resuspension following the procedures reported for MMV (Lastra & Acosta, 1979; McDaniel, Ammar & Gordon, 1985). Several combinations of polyethylene glycol (PEG) and NaCl were tested as an alternative to differential centrifugation (Hsu & Black, 1973; Jackson & Christie, 1977). PEG at 6% and NaCl at 0.1 M gave satisfactory preparations, as determined by electron microscopy. Celite filtration prior to sucrose gradient centrifugation was essential to reduce contamination by host plant components. The majority of the particles following quasi-equilibrium zonal density gradient centrifugation appeared bullet-shaped, but a small proportion of pleomorphic particles were also observed. No host contaminants were detectable by electron microscopy in the purified virus preparations.

Serology

The antisera had a titre of 1/2650 in the precipitin ring interphase test. In agarose double-diffusion tests, the undiluted antiserum consistently gave a single precipitin line with virus in infected sorghum leaf extracts, but not with healthy leaf extracts. In immuno diffusion tests, MMV-RN antiserum gave a strong confluent precipitin line, whereas the MMV-HI an-

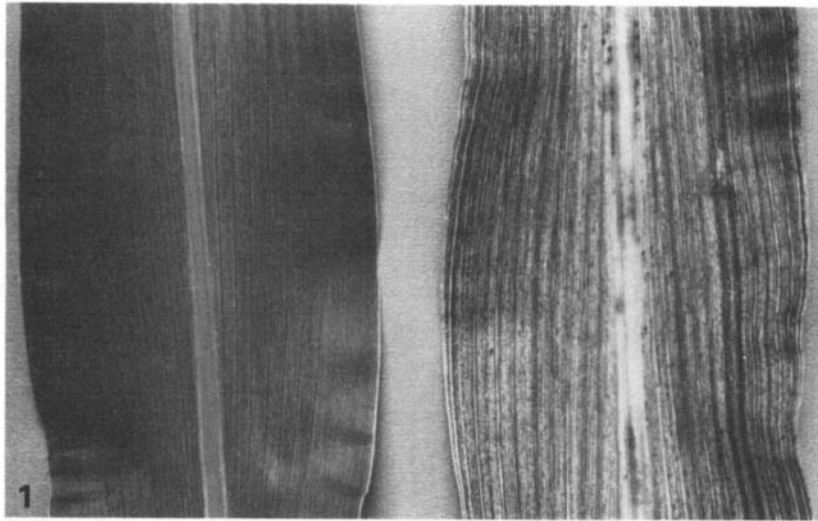


Fig. 1. Symptoms of maize mosaic virus, sorghum isolate (MMV-S), in sorghum leaf. Healthy leaf on left.

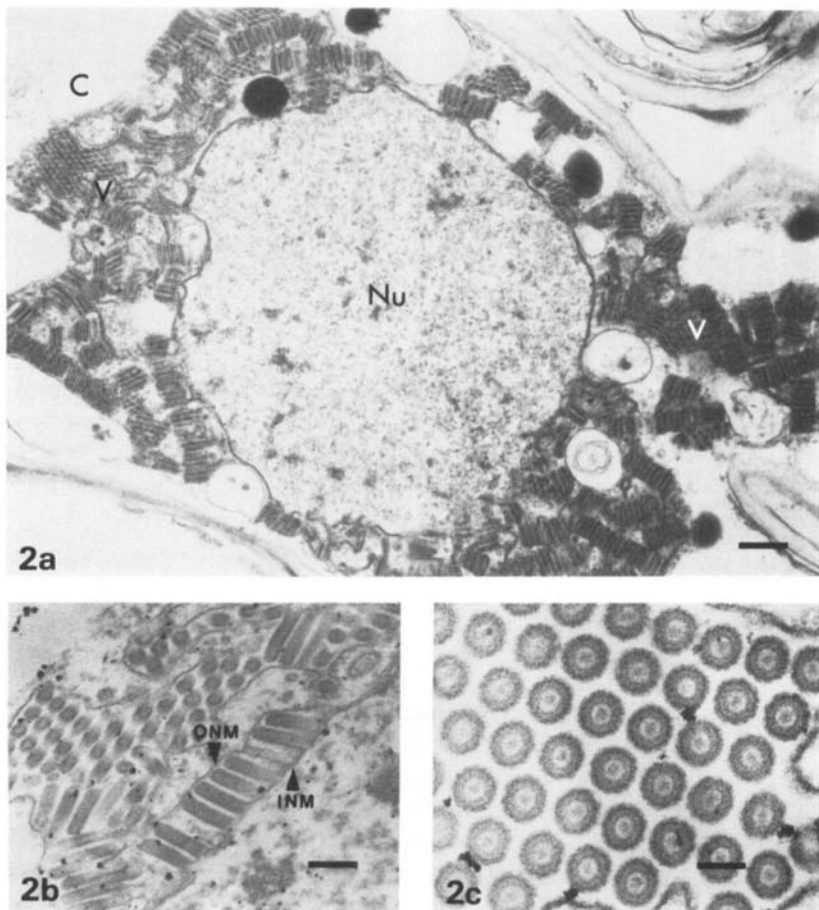


Fig. 2. MMV-S particles in infected sorghum cells: *a*. virus particles (V) accumulated in the cytoplasm (C) around the nucleus (Nu) of a mesophyll cell. Bar represents 500 nm; *b*. virus particles between the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). Bar represents 175 nm; *c*. virus particles in cross-section. Bar represents 60 nm.

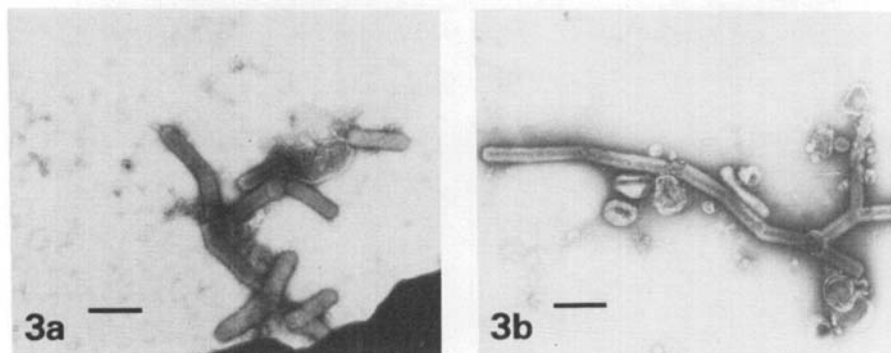


Fig. 3*a*. MMV-S particles in leaf dip preparations fixed with 1% glutaraldehyde and stained with 1% aqueous uranyl acetate, pH 4.0. Bar represents 200 nm; *b*. End-to-end aggregation of virus particles. Bar represents 200 nm.

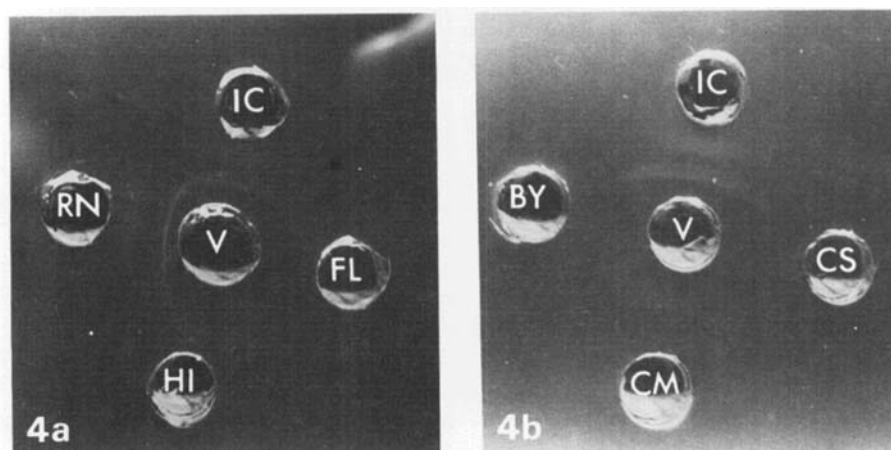


Fig. 4. Immunodiffusion reactions of MMV-S: *a*. with various MMV isolates — Reunion (RN), Hawaii (HI), and Florida (FL); *b*. with barley yellow striate mosaic virus (BY), cereal chlorotic mottle virus (CM), and cynodon chlorotic streak virus (CS). IC = the MMV-S undiluted antiserum. V = sap from MMV-S infected sorghum leaf.

tiserum gave a very weak precipitin line. The MMV-FL, CCSV, CCMV, and BYSMV antisera failed to show any reaction (Plate 2, fig. 4a,b).

ELISA

In DAC-ELISA, the homologous antiserum at a 10^{-4} dilution detected the virus in infected sorghum extracts diluted up to 10^{-5} . In comparative ELISA tests (Table 1), the MMV-S cross-reacted strongly with the MMV-RN antiserum, and weakly with MMV-HI and MMV-FL antisera. The CCSV and CCMV antisera reacted positively with MMV-S. Additionally, it reacted weakly with BYSMV antiserum. These three antisera (CCSV, CCMV, BYSMV) gave relatively high absorption values with healthy sorghum leaf extracts.

SDS-PAGE

Four major virus proteins (G, N, M_1 and M_2) in order of decreasing molecular weight, according to the international nomenclature for rhabdovirus proteins (Wagner *et al.* 1972), were

Table 1. Determination of serological relationships of sorghum isolate of maize mosaic virus (MMV-S) by direct antigen coating form of enzyme-linked immunosorbent assay

Antiserum dilution +	Dilution of sorghum leaf extracts *:					
	Healthy			Infected		
	10 ⁻²	10 ⁻³	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
MMV-S						
10 ⁻²	0.28**	0.31	2+	2+	1.87	0.78
10 ⁻³	0.13	0.12	1.53	1.52	1.23	0.49
10 ⁻⁴	0.05	0.05	0.99	0.91	0.77	0.26
MMV-RN						
10 ⁻²	0.17	0.13	1.60	1.45	1.37	0.65
10 ⁻³	0.08	0.06	1.01	0.94	0.79	0.33
10 ⁻⁴	0.05	0.05	0.39	0.39	0.35	0.15
MMV-HI						
10 ⁻²	0.33	0.25	1.46	1.54	1.40	0.77
10 ⁻³	0.16	0.13	0.77	0.75	0.69	0.33
10 ⁻⁴	0.08	0.07	0.32	0.31	0.28	0.14
MMV-FL						
10 ⁻²	0.14	0.13	0.45	0.41	0.40	0.23
10 ⁻³	0.06	0.05	0.17	0.15	0.14	0.08
10 ⁻⁴	0.05	0.05	0.09	0.08	0.07	0.04
CCSV						
10 ⁻²	0.95	0.91	1.78	1.75	1.62	0.92
10 ⁻³	0.45	0.39	1.02	1.00	0.88	0.43
10 ⁻⁴	0.14	0.13	0.33	0.32	0.31	0.15
CCMV						
10 ⁻²	0.81	0.60	1.12	1.10	1.03	0.60
10 ⁻³	0.42	0.35	0.60	0.54	0.51	0.28
10 ⁻⁴	0.15	0.13	0.21	0.20	0.19	0.13
BYSMV						
10 ⁻²	0.37	0.33	0.54	0.52	0.48	0.29
10 ⁻³	0.20	0.17	0.29	0.28	0.26	0.15
10 ⁻⁴	0.10	0.08	0.13	0.13	0.10	0.10

+ Details described in the text.

* Dilutions are based on the original weight of tissue.

** The mean A_{405} nm value for three replicates. The mean A_{405} nm value for buffer control = 0.08.

consistently resolved by SDS-PAGE on 10% polyacrylamide gels (Plate 2, fig. 5a). The molecular weights of individual proteins were estimated in 10% gels by plotting the mobilities of standard proteins against the \log_{10} of their respective molecular weights. Average molecular weights from five gels were estimated to be 70 000 for G protein, 59 000 for N, 32 000 for M_1 , and 28 000 for M_2 .

Electro-blot immunoassay

When the polypeptides separated by SDS-PAGE, were transferred electrophoretically to nitrocellulose and probed with homologous, MMV-RN, MMV-HI, and CCSV antisera, only G and N proteins could be detected (Plate 2, fig. 5b). With BYSMV antiserum, no reaction was observed with any of the polypeptides.

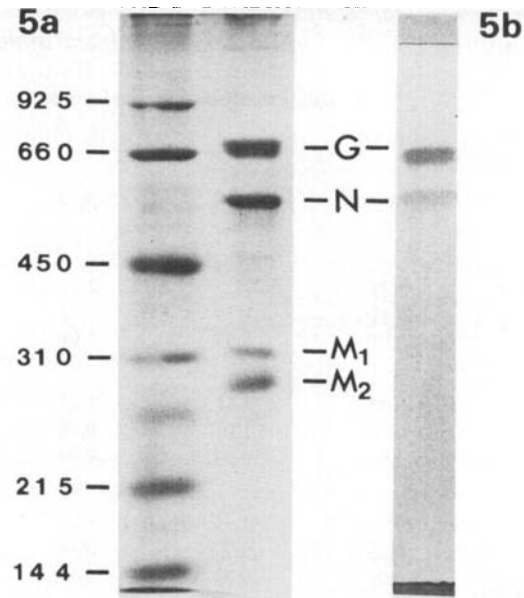


Fig. 5a. SDS-polyacrylamide slab gel showing polypeptides of MMV-S. S shows the molecular weight markers and V shows MMV-S virus proteins; b. Immuno-blot of the MMV-S proteins from a. after probing with homologous antiserum.

Discussion

Our evidence shows that MMV infects sorghum, and we prefer to name it the MMV-S isolate. From our disease surveys it appears that MMV is economically important in sorghum. This report is the first to document fully the occurrence of a rhabdovirus in a cereal crop in the Indian sub-continent. Diseases considered to be caused by rhabdoviruses in cereals have been reported from the Indian subcontinent; the causal viruses, however, were not fully characterised. Thus we are unable to compare our results with previous findings from the region.

MMV was first reported from Hawaii in 1921 (Kunkel, 1921). It is considered to be an economically important disease of maize (Brewbaker, 1981) in the tropics and subtropics. We are not aware of any report, however, that suggests that MMV to be economically important on sorghum, as was observed in our survey (data not shown). Sorghum stunt mosaic virus (SSMV), a rhabdovirus reported to occur naturally on sorghum, is transmitted by *Graminella sonora* (Mayhew & Flock, 1981), but not by *P. maidis*. Additionally, SSMV and MMV are not identical (Mayhew & Flock, 1981).

Maize crops grown adjacent to heavily infected sorghum crops were surveyed for MMV-S. When its incidence in sorghum exceeded 50%, it occurred in less than 1% of maize plants in adjacent fields. Several apparently healthy looking maize plants were tested by ELISA with negative results. The *P. maidis* collected from sorghum fields bred poorly on maize and thus we used sorghum to raise healthy colonies. We have not performed transmission tests with viruliferous *P. maidis* to determine susceptibility of maize to the MMV-S from sorghum, but are currently studying the host range of the MMV-S isolate.

The purification method developed gave preparations devoid of host contaminants as judged by electron microscopy, and polypeptide composition of virions in gel electrophoresis. In both the double diffusion and DAC-ELISA tests, MMV-S reacted strongly with the MMV-RN, weakly with MMV-HI and MMV-FL isolates. However, the titre of the MMV-FL antiserum was low (B.W. Falk, personal communication). In DAC-ELISA, the MMV-S also reacted strongly with antisera to CCSV and CCMV and weakly with BYSMV antiserum. Electro-

blot immunoassay results also showed positive reaction with antisera to other MMV isolates, CCSV and CCMV but not with BYSMV. Lockhart, Khaless, El Maataoui & Lastra (1985) reported that CCMV is not serologically related to the MMV-HI isolate nor to CCSV. Thus, we are unable to interpret results in which MMV-S cross reacted with CCMV and CCSV. Contamination of sorghum plants with CCSV and CCMV is unlikely because *P. maidis* is not a vector of these two viruses.

The cytopathology of MMV-S (which includes accumulation of virions in the perinuclear space, budding of virions through the inner nuclear membrane, and infrequent presence of particles in the nucleus) resembles that reported for other MMV isolates (Bradfute & Tsai, 1983; Lastra, 1977; Martelli, Russo & Malaguti, 1975; McDaniel *et al.*, 1985; Ammar, Gomez-Luengo & Gordon, 1987). Like the MMV-HI isolate (McDaniel *et al.*, 1985), the majority of the particles were bullet-shaped in glutaraldehyde-fixed and uranyl acetate stained preparations. However, in unfixed preparations, the majority of the particles were pleomorphic. The bullet-shaped particles (208 × 66 nm) were similar in size to other MMV isolates (Falk & Tsai, 1983; Jackson, Milbrath & Jedlinski, 1981; McDaniel *et al.*, 1985).

Analysis of MMV-S proteins by SDS-PAGE revealed four major polypeptides, G, N, M₁ and M₂. In contrast to our MMV-S isolate from India, the MMV-FL isolate had only three major polypeptides (Falk & Tsai, 1983). However, recently Gomez-Luengo & Gordon (1987) reported five (L, G, N, M₁ & M₂) proteins for MMV-HI, MMV-FL, and a Costa Rican isolate of MMV. Although the molecular weights of polypeptides of MMV-FL and MMV-S isolates differed slightly, the differences were within the error limits expected for the technique. In electro-blot immunoassay utilising various MMV antisera, CCSV and CCMV, only G and N proteins were detected. However, BYSMV antiserum failed to react with any of the polypeptides. Thus the major serological activity detected in our studies is related to the G and N proteins and confirms observations reported for other plant rhabdoviruses (Adam, Chagas & Lesemann, 1987). Our data indicate that MMV-S closely resembles plant rhabdoviruses classified in subgroup II (Jackson, Francki & Zuidema, 1987; Peters, 1981).

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