PURIFICATION OF UNAGGREGATED SUGARCANE MOSAIC VIRUS DIRECTLY FROM SUGARCANE LEAVES

By K. M. HARBORNE-RUTHERFORD, R. A. BAILEY

South African Sugar Association Experiment Station, Mount Edgecombe 4300 South Africa

and J. V. DA GRACA

Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg 3201, South Africa

Abstract

A practical method has been developed for the rapid purification of unaggregated sugarcane mosaic virus (SCMV) directly from infected sugarcane leaves in sufficient quantities for antiserum production. The antiserum produced to the purified virus showed no reaction with sap from healthy sugarcane leaves in microprecipitin tests or on agar diffusion plates and it has been successfully used in the enzyme-linked immunosorbent assay (ELISA) technique and in immunosorbent electron microscopy (ISEM) to detect SCMV. A press for extracting the sap from leaves for virus studies is described.

Introduction

Sugarcane mosaic is the most important virus disease of sugarcane in South Africa. The disease is widespread in the coastal hinterland and high altitude areas of Natal, where a large percentage of the sugarcane crop consists of susceptible varieties. In these areas, mosaic can spread rapidly in fields of susceptible varieties in the early stages of growth during the summer months (Bailey and Fox[1]). Sugarcane mosaic virus (SCMV) is a member of the serologically related group of viruses called potyviruses. These viruses are flexuous, rod-shaped particles, about 750 nm long in the case of SCMV, and are all transmitted in a non-persistent manner by aphids (i e the aphid remains infective for only a short time) and experimentally by mechanical inoculation of sap (Hollings and Brunt[10] Pirone[13]).

At least two strains of SCMV occur in sugarcane in South Africa. Strains SCMV-A and SCMV-D have been identified, with SCMV-D being by far the more common. From reactions on differential hosts, the strain occuring in the widely grown, susceptible variety NCo 376 appears to be a substrain of SCMV-D (Gillaspie[7,8]).

To investigate the epidemiology of mosaic, a more reliable and sensitive method of detection than the visual observation of symptoms on the leaves of known and possible host plants is desirable. The enzyme-linked immunosorbent assay (ELISA) technique (Voller *et al*[15]) provides such a method. There are a number of variations of this technique (Banttari and Goodwin[2]; van Regenmortel[14]), which is based on the immunogenic reaction of animal antibodies with specific foreign proteins (antigens). ELISA is sufficiently sensitive to detect as little as 10 ng of virus per sample, which makes it a very useful technique for virus research (Clark and Adams[3]).

To use ELISA for the detection of SCMV, the virus must be purified and then injected into a suitable animal to produce an antiserum containing the specific antibodies. The type of animal chosen for producing antisera to plant viruses depends largely on the quantity required, the most commonly used animal is the rabbit.

With some methods of purifying SCMV, and the closely related maize dwarf mosaic virus (MDMV), the purified preparation is contaminated by ribulose bis-phosphate (protein fraction 1). This is undesirable because it results in a poor quality antiserum which is not sufficiently specific for diagnostic tests (van Regenmortel[14]). If such a serum is used in an ELISA test, it will result in a high 'background' reaction, making it difficult to distinguish between negative and positive reactions. Langenberg[11] successfully purified MDMV-B by using the detergent Triton X-100 to reduce ribulose bis-phosphate to low or undetectable levels. The procedure reported in this paper for purifying SCMV-D from sugarcane is a modification of Langenberg's method.

Different methods of purifying SCMV have been reviewed by Gillaspie.[6] These and other reported methods are all based on the use of inoculated maize or sorghum as an intermediate source of the virus. These host plants are used because their leaves are larger and softer than those of sugarcane, and thus the virus is easier to extract and it is also present in higher concentrations. However, reports of up to 30% transmission of MDMV in maize seed (von Wechmar *et al*[16]) indicate that maize should not be used as an intermediate host for bulking-up SCMV before purification due to possible contamination by other viruses. It was therefore decided to purify the virus directly from sugarcane.

Using ELISA, strains of SCMV can be identified as belonging to one of the following groups: A, B, D, K; H, I; E (Gillaspie and Mock[9]), but they cannot be differentiated further. With careful preparation of antisera to pure strains of SCMV purified directly from sugarcane, it may be possible to distinguish between strains within these groups.

Materials and Methods

Source of virus

SCMV-D was obtained from naturally infected NCo 376 collected at Tanhurst Estate in the Dumisa area of Natal. This isolate of SCMV has been identified by Gillaspie[8] as a possible variant of strain D by means of the reactions of differential hosts. Infected stalks of NCo 376 were cut into single-budded setts and planted in trays in a glasshouse to provide a source of infected material. When the plants were approximately 50 cm tall, leaves showing marked symptons of mosaic were collected; these were usually the first two unfolded leaves and the spindle. This material was often supplemented with freshly collected, infected young leaves of NCo 376 from the same field as the original collection.

One kilogram of young, infected leaf tissue was used for each purification. Whole leaves were cut into pieces approximately 1 cm long, which were then weighed into lots of 100 g. Each lot of leaf material was placed into a thick polythene bag and pounded with a hammer to disrupt the

tissue. The bruised tissue was then soaked in cold (4°C) 0,01 M phosphate + 0,3% 2-mercaptoethanol buffer (pH 7,0) for 5 min using 2 ml buffer g^{-1}. The liquid was then decanted and left to stir on a magnetic stirrer.

Extraction of leaf fluids

A hydraulic press was designed to extract sap from the sugarcane leaves. The leaf press was constructed from a 200 kN hydraulic ram mounted on a metal frame (Figure 1). Pressure is applied to the tissue by means of a plunger sliding in a cylindrical sleeve which is mounted on a reservoir (Figure 2). The reservoir is fitted with a spout from which the expressed juice (buffer plus tissue fluids) flows into a collecting vessel. The three parts of the tool, plunger, sleeve and reservoir, were machined to close tolerances from stainless steel (Figures 3 and 4). The plunger fits closely in the cylinder (clearance = 0,04 mm); this effectively prevents fluids being forced up past the plunger during compression.

Approximately 50 g of leaf tissue are placed in the sleeve, already mounted on the reservoir, and the plunger is fitted. The ram is then operated causing the plunger to slowly exert a pressure of up to approximately 40 MPa on the tissue. This pressure ruptures the cells and releases the sap, which is forced out of narrow grooves cut in the base of the sleeve. The sap accumulates in the well of the reservoir, and runs off for collection. Most of the sap is expressed at pressures of 4 to 20 MPa. The clearance of 6 mm between the base of the sleeve and the floor of the reservoir (Figure 3) prevents sap being sucked back into the leaf tissue after pressure is released. Extracted sap is free of most cell debris and need not be filtered before further use.

Approximately 36 ml of leaf fluids were expressed from every 100 g of fresh leaf tissue, which had an initial moisture content of approximately 75%.

Purification of virus

The expressed liquid was pooled with the buffer decanted earlier and to every 50 ml of the mixture 1 ml of 2,0 M calcium chloride ($CaCl_2$) and 2 ml of 2,0 M dipotassium hydrogen phosphate (K_2HPO_4) were added. This liquid was then centrifuged for 10 min at 8 500 rpm (11 700 g) in a Sorvall GSA rotor at 5°C in 250 ml bottles. The pale green-brown supernatant was decanted and to every 87,5 ml of this 12,5 ml of a mixture of 4% (v/v) Triton X-100 (alkylphenoxypolethoxyethanol) and 48% (w/v) polyethylene glycol (PEG MW 8000) was added, to make the final concentrations of these compounds 0,5 and 6% respectively. Ten aliquots prepared from the 1 kg of leaf tissue were pooled and left to stir for 2 to 3 h on ice. The resulting precipitate was removed by low speed centrifugation for 10 min at 8 500 rpm in the Sorvall GSA rotor at 5°C. The white pellet obtained was resuspended in 40 ml of 0,01 M TACm buffer (0,01 M TRIS, 0,005 M citric acid, 0,01 M 2-ME, 0,1% Triton X-100 with the pH adjusted to 7,2 with 0,2% 1,3-propane diamine). This was then centrifuged for 10 min at 9 000 rpm (8 700 g) in a Sorvall SS-34 rotor. The light yellow-green, opaque supernatant was layered on 5 ml of a 30% sucrose solution in TACm buffer in 10 ml tubes in a Spinco S75 Ti rotor and was given a high speed centrifugation for 1 h at 33 000 rpm (68 000 g) at 4°C in a Beckman L8-M ultracentrifuge in order to pellet the virus. The small clear pellets were resuspended in 3 ml of 0,01 M TACm buffer and centrifuged for 10 min at 9 000 rpm in a Sorvall SS-34 rotor to remove the insoluble material. The supernatant was layered onto linear 100 to 400 mg ml^{-1} sucrose density gradients buffered with 0,01 M TACm buffer. The gradients were prepared using the simple Perspex apparatus referred to by Noordam.[12] The density gradients were centrifuged for 2 h at 23 000 rpm (73 000 g) in a Spinco SW25.1 swinging bucket rotor at 4°C. The fraction of the gradient containing the virus was observed by shining a light beam up through the centrifuge tube and the virus could be detected as an

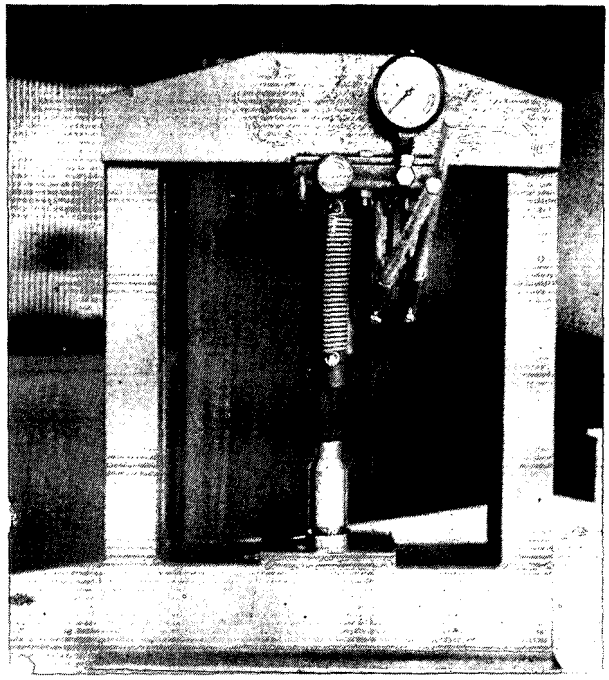

FIGURE 1 Hydraulic ram press for extracting fluids from leaf tissue

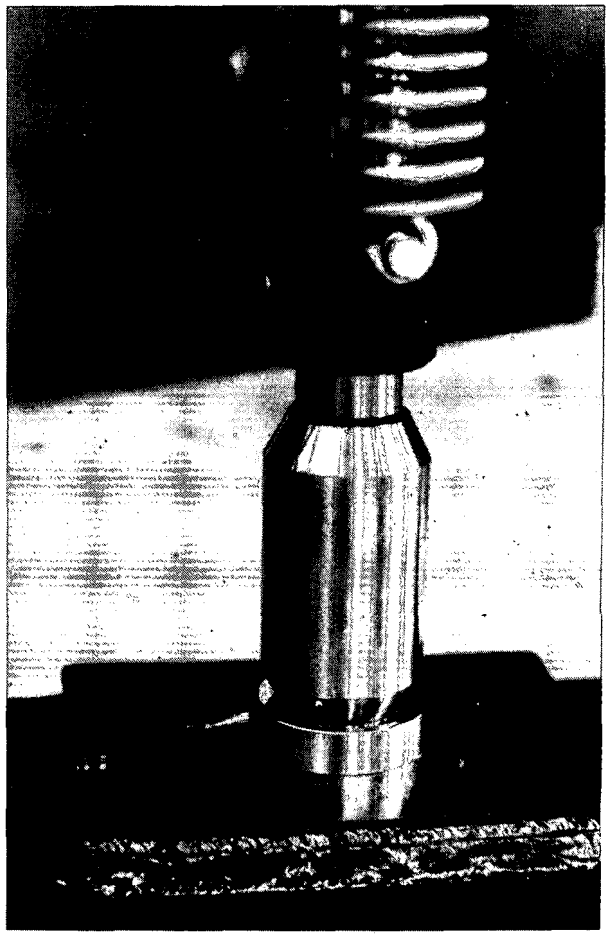

FIGURE 2 The stainless steel tool with sap being expressed

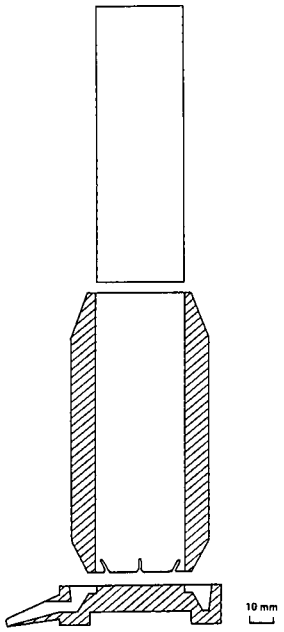

FIGURE 3 Cross-section of the stainless steel tool used in the hydraulic leaf press

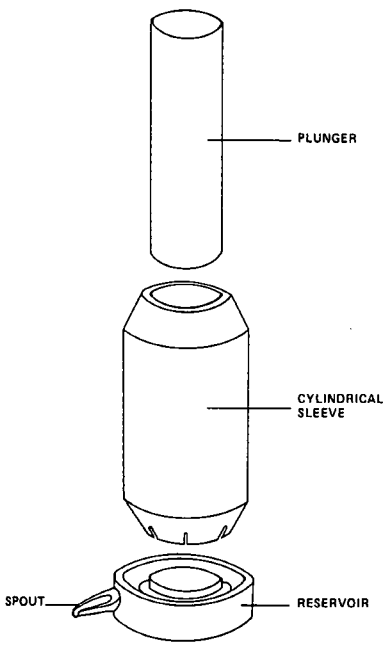

FIGURE 4 Isometric drawing of plunger, sleeve and reservoir

opalescent, light-scattering band. The virus bands were removed by means of a syringe with the last cm of the needle bent at 90°. The sucrose was removed from the virus preparation by a high-speed centrifugation for 1 h at 33 000 rpm (68 000 g) in the S75 Ti rotor. The clear pellets were finally pooled and resuspended in 4 ml 0,01 M TRIS buffer.

The concentration of virus in the final buffer was measured with a spectrophotometer and converted into mg ml^{-1} using the UV extinction co-efficient E260/E280 of 1,21 to 1,24 for SCMV (Gillaspie[6]).

Drops of the diluted virus suspension (1 : 10) were placed on Formvar-coated grids, stained with 1 % potassium phosphotungstate (pH 7,0) and examined in a JEOL 100-CX electron microscope.

Antiserum production

Two rabbits were injected intramuscularly with the purified virus preparation emulsified with Freund's complete adjuvant. The initial immunization consisted of six injections per rabbit. Four injections per rabbit were given on each of five occasions, 7, 14, 28, 90 and 105 days after immunization, using freshly purified virus. Approximately 30 ml of serum was taken from each rabbit 118 days after immunization. The antiserum was tested using the gel diffusion method to test for reactions to ribulose bis-phosphate from healthy sugarcane.

Immunosorbent electron microscopy

Formvar-coated grids were floated on a 1 : 10 dilution of the antiserum for 1 h (Derrick and Brlansky[4]). After washing in 0,05 M TRIS-HCl buffer (pH 7,2), the grids were floated for 6 h on drops of virus extract prepared by grinding infected sugarcane leaf material with a pestle and mortar in 0,01 M phosphate buffer (pH 7,2; 0,3 % 2-mercaptoethanol). The grids were washed five times in extraction buffer, then in distilled water, dried, stained with 2 % potassium phosphotungstate (pH 7,0) and viewed in the electron microscope. Control grids, not coated with antiserum, were also prepared.

Results and Discussion

Small amounts of pure sugarcane mosaic virus were obtained by this purification procedure. The concentration of virus in the final buffer ranged from 0,1 to 0,25 mg ml^{-1}, which is equivalent to a virus yield from infected sugarcane leaf tissue of 0,4 to 1,0 mg kg^{-1}. This is a relatively low yield when compared with the 5 to 28 mg kg^{-1} for MDMV obtained by Langenberg[11] from infected maize leaves and with the yields of some other potyviruses (Hollings and Brunt[10]).

The purified virus contained no detectable host protein. This was demonstrated by the lack of precipitation bands forming in gel diffusion slides. The titre of the antiserum according to the microprecipitin test was 1 : 256.

When examined under a transmission electron microscope, the purified virus preparation was seen to consist of unaggregated flexuous rods of SCMV (Figure 5). This indicates that the virus retained is integrity throughout the purification procedure and was therefore in a suitable condition to be inoculated into rabbits to produce antiserum.

The antiserum to SCMV-D produced from virus purified directly from sugarcane has been used successfully to develop a highly sensitive ELISA technique for the rapid detection of SCMV. This technique is based on the dot-ELISA method using nitrocellulose membranes, described by Banttari and Goodwin.[2] The relatively low titre of 1 : 256 was adequate for use in dot-ELISA tests, although antisera with higher titres were used for ELISA by Devergne *et al*[5] and Clark and Adams.[3] The success with the dot-ELISA method can probably be attributed to the increased binding efficiency of the nitrocellulose membrane compared with the conventional microtitre plates used in other methods of ELISA. The antiserum was also effective at trapping SCMV particles from crude infected sugarcane sap where the virus concentration was known to be low (Figure 6).

The extraction and purification of SCMV from sugarcane was facilitated by the use of the specially designed leaf press. Initial attempts to purify the virus were carried out using a blender to break up the sugarcane leaf tissue, but it was difficult to maintain the buffer and extract at a low temperature. The homogenized tissue also had to be filtered through cheesecloth and it was never possible to remove as

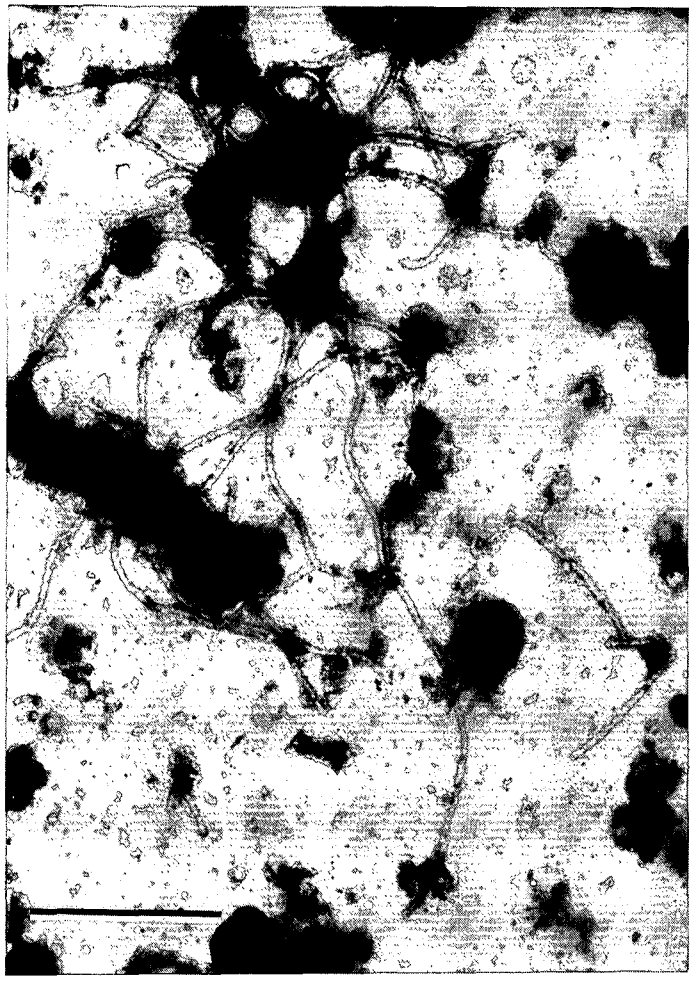

FIGURE 5 Electron micrograph of SCMV particles purified from infected sugarcane (bar is 1 000 nm)

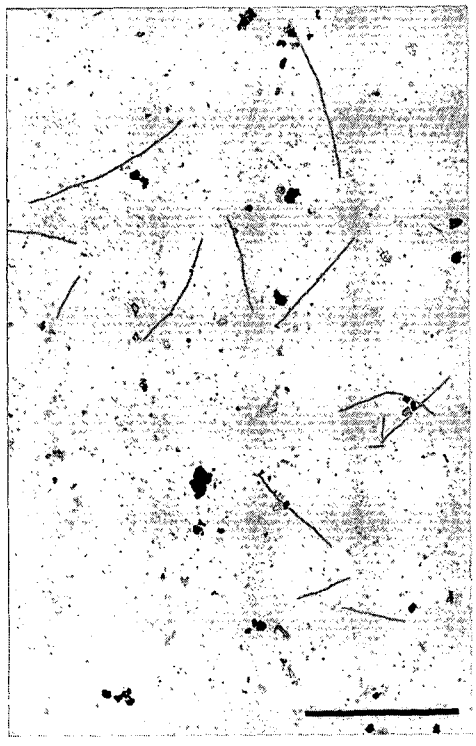

FIGURE 6 Electron micrograph of SCMV particles trapped on Formvar-coated grids by 1 : 10 dilution antiserum (bar is 500 nm).

much of the sap as it was when using the press. The need for filtering also increased the time taken to purify the virus when the leaf tissue was blended. The leaf press facilitates the rapid extraction of large amounts of sap and also enables the tissue to be maintained at a low temperature throughout the extraction procedure. This minimises the possibility of enzymatic breakdown of the virus particles.

The initial step of cutting the leaves into small pieces, bruising them and then leaving them to soak in buffer improved the efficiency of virus extraction, probably because the buffer could then penetrate the tissue where it protected the virus from oxidizing enzymes. The buffer also facilitates the diffusion of cytoplasm containing the virus by disrupting the plant cell membranes. The initial bruising also increased the volume of sap expressed by the press, from approximately 26 to 36 ml 100 g^{-1} of leaf tissue.

The press can also be used for expressing sap from small quantities of leaf material, for example when testing numerous samples for the presence of virus in crude sap extracts. Using a scaled-down model of the stainless steel tool, with a 50 × 25 mm diameter plunger, sap can be rapidly expressed from 1 to 5 g tissue. The tool is easily disassembled and cleaned with methanol between samples.

Conclusions

The purification procedure for SCMV reported here is rapid, relatively simple, and enables large quantities of infected sugarcane leaves to be processed. The hydraulic press is a major factor contributing to the success of the procedure. It may also be possible to improve the efficiency of the purification procedure further, by using the antiserum already produced to identify any steps in the extraction and purification where significant quantities of virus are lost.

The antiserum to SCMV-D purified directly from sugarcane was of a high quality, and showed no visible background reaction with sap from healthy plants in ELISA tests. This increases the sensitivity of the test. The satisfactory titre of the antiserum indicates that it is not necessary to immunize rabbits with large quantities of SCMV antigen, provided it is a pure preparation.

SCMV is an unstable virus, which readily degrades into small fragments. It is known that the reactivity of antibodies produced to whole virus particles differs from that of antibodies produced to virus fragments. This purification procedure resulted in a final virus preparation that consisted mainly of whole particles and this contributed to the sensitivity of ELISA for the rapid detection of SCMV in crude sap extracts.

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