An efficient method for purification of soybean mosaic virus and production of a highly specific polyclonal antiserum.

Abstract: The soybean mosaic virus (SMV) is considered to be the most important virus for soybean, Glycine max, in the world. An isolate of SMV obtained from seeds of infected soybean 'Bragg' was purified and a highly specific polyclonal antiserum was obtained for use in double highest yield of the best virus purified preparation was estimated to be around 30 mg of virus per Kg of infected soybean leaf tissue. The purified SMV preparation was highly in soybean 'IAC-2'. The polyacrylamide gel electrophoresis with sodium dodecyl sulfate (PAGE-SDS) analysis of a freshly purified preparation revealed a main protein component with an estimated molecular weight (MW) of 32 k. An obtained polyclonal antiserum was reactive in SDS-immunodiffusion and indirect-ELISA tests with saps of infected plants without showing any reaction with extracts of non inoculated plants in double diffusion. Purified IgG showed a good concentration and was also very specific to
the virus in indirect ELISA and western blotting tests. This seems to be the first specific antiserum produced for SMV in Brazil. As it showed to be very specific and useful for different serological tests, including to detect the presence of the virus in infected seeds, it will be of great value for diagnosis and research purposes.

INTRODUCTION

Although introduced in Brazil in 1882 (13) the soybean, *Glycine max* (L.) Merril, became one of the most economically important crop for the Country. After its first observations in the "Instituto Agronômico de Campinas", São Paulo, and its introduction in the Rio Grande do Sul in 1914 (4), the soybean started to constitute the main cash crop in South of Brazil. The great internal and external demand for its grains and derivatives stimulated its expansion in regions of lower latitude, constituting better alternative then rice for "cerrado" in the Central Brazil. Brazil is today the second greatest producer and exporter of soybean in the world.

The expansion of soybean all over the world has contributed for dispersion of several disease causal agents is number and severity. More than a hundred of different pathogens are capable to cause disease in soybean, and around 35 are economically importante (21). Among the different pathogens that infect soybean, the viruses have constituted limiting factors for the crop production. More than 20 viruses are listed in the literature as found naturally infecting soybean (21;15;17). Around 13 different viruses were reported infecting naturally or experimentally soybean in Brazil, including the soybean mosaic potyvirus (SMV) (10;3), bean pod mottle comovirus (BPMV) (5); tobacco streak ilarvirus (TSV) (10); southern bean mosaic sobemovirus (SBMV) (11), bean rugose mosaic comovirus (BRMV) (12); cowpea severe mosaic comovirus (CpSMV) (6); and a possible strain of cowpea aphidborne mosaic potyvirus (CpAMV) in Ceará (22).

Considering the world dispersion, facility of seed and aphid transmission, as well as the disease severity and the decrease in crop production, the SMV is considered to be most important virus for soybean in the world (21;15). The SMV was the first virus found infecting soybean in the United Sates and it is distributed all over the world. The virus is responsible for reduction of weight and number of seeds in infected plants. Besides the reduced size the infected seeds are usually spotted and present low germination (1;2). The virus belongs to the genus *Potyvirus*, family *Potyviridae* (20).

In the present paper an isolate of SMV obtained from seeds of infected soybean 'Bragg' produced in the State of Paraná was purified and a highly specific polyclonal antiserum was obtained for use in double immunodiffusion and enzyme linked immunosorbent assay and western blotting tests.
MATERIAL AND METHODS

1 - Virus Isolation and Maintenance

The Virus was recovered from infected seeds originated from the National Soybean Research Center, at Londrina, Paraná. Seeds from SMV infected plants of soybean 'Bragg' grown in the State of Paraná were sown at greenhouse conditions in the Plant Virus Laboratory at the Federal University of Ceará. The plants showing mosaic symptoms were tested against antiserum specific for SMV (D.E. Purcifull, University of Florida) and the virus was mechanically transferred to soybean 'IAC-2'. The inoculated plants were maintained at greenhouse conditions with temperature varying from 26°C (night) to 38°C (day) and the virus was periodically transferred to health new plants as the infected get old. The recovered virus was identified as the G1 strain of SMV on the basis of the symptomatological reactions induced in differential soybean genotypes at the National Soybean Research Center Paraná (2), according to Cho & Goodman (9).

2 - Virus Purification and Antiserum Production

The virus isolate was increased in soybean 'IAC-2' since this cultivar presented good reaction to the virus infection, as well as good quantity of infected leaves that presented a lot of cytoplasmic inclusions, good indications of high concentration of virus. A lot of approximately 200 pre-germinated seeds were planted in sterile potted soil at greenhouse conditions at the Federal University of Ceará and the virus was mechanically inoculated 4 days later in the cotyledonary leaves. The systemically infected leaves were harvested 15 days after inoculation for virus purification.

The virus purification was based on the method described by Calvert & Ghabrial (8) with important modifications to avoid possible loss of viruses due to irreversible virus particles aggregations. Virus-infected leaves were ground in a blender, in the presence of 0.16 M sodium phosphate buffer, pH 7.5, containing 0.2 M trisodium citrate, 0.01 M EDTA, 0.1% of sodium diethylthiocarbamate and 0.5% of 2-mercaptoethanol. The obtained leaf extracts were strained through two layers of cheesecloth and approximately 7% of n-butanol was added to the filtrate and the mixture was stirred overnight at 4°C. The mixture was centrifuged at 8,000 g for 10 min and the pellet was discarded, and the supernatant containing the virus was layered on a cushion of 30% sucrose prepared in 0.05 M sodium phosphate buffer, pH 7.6, containing 0.01 M EDTA and 0.5% Triton X-100, and centrifuged at 90,000 g for 3.5 h. The precipitated virus particles were resuspended in the cushion buffer and further purified by equilibrium centrifugation at 120,000 g for 18 h in 30% (w/w) CsCl prepared in the same buffer. The virus zone located at 2 - 4 cm from the bottom of the tube was collected, diluted with the same buffer and clarified by a centrifugation of 10,000 g for 10 min. The
partially purified virus was re-concentrated by a centrifugation of 85,000 g for 1.5 h, and resuspended in 20 mM Tris buffer, pH 8.2.

The purified virus preparation was evaluated biologically by mechanical inoculation in soybean 'IAC-2' and spectrophotometrically in UV-variant DMS spectrophotometer. The virus concentration in the purified preparation was determined using a $E_{600} = 2.8$ determined by Stace-Smith & Tremaine (23) for potato virus Y (PVY) the type member of the potyvirus group.

3 - Polyacrylamide Gel Electrophoresis of Virus Capsidial Protein

The Molecular weight of the SMV capsidial protein was determined by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (PAGE-SDS) according to the method described by Sambrook et al. (19).

The purified virus preparation was diluted at 1:50; 1:100 and 1:200 in the sample buffer (Tris-HCl 50 mM pH 6.8; dithiothreitol 100 mM; SDS 2%; bromphenol blue 0.1% and glycerol 10%), heated at 100°C for 3 min and centrifuged at 3,000 g for 2 min. Aliquots of 20 l from each dilution were applied per well in the concentration gel, which contained 5% of acrylamide (1.4 ml of distilled water, 0.33 ml of a solution containing 29% of acrylamide and 1% of acrylamide and 1% of bis-acrylamide; 0.25 ml of Tris 1M pH 6.8; 0.02 ml of SDS at 10%; 0.02 ml of ammonium persulphate at 10% and 0.002 ml of tetramethylethlenediamine-TEMED). The gel for separation was composed of 12% of acrylamide (1.6 ml of distilled water; 2 ml of solution containing 29% of acrylamide and 1% of bis-acrylamide; 1.3 ml of Tris 1.5M pH 8.8; 0.05 ml of SDS at 10%; 0.05 ml of ammonium persulphate at 10% and 0.002 ml of TEMED). The polymerization of acrylamide and bis-acrylamide in the presence of ammonium persulphate and TEMED occurred at room temperature.

The electrophoresis was performed at a vertical discontinuing BRL system, model MINI-V-8.10, at room temperature, using the running buffer (Tris 25mM, glycine 250mM and SDS 0.1%), at a constant voltage of 115V during 2-3 h. The following protein markers were used: bovine serum albumin (MW = 66k), ovalbumin (MW = 45k), pepsin (MW = 34.7k), trypsinogen (MW = 24k) and B-lactoglobulin (MW = 18.4 k). The protein bands were located in the gel by staining with a solution of 0.25% comassie blue R250, 45% methanol and 10% glacial acetic acid, during 1 h. The excess of stain was removed from the gel by washing it at solution of 7% glacial acetic acid and 30% methanol.

3.4 - Antiserum Production and IgG Purification

A five months old New Zealand white rabbit was immunized with untreated virus purified preparation used as antigen was emulsified with an equal volume of
Freund's incomplete adjuvant (Difco) and a series of three foot pad injections was given to the rabbit according to Lima et al. (18), with a week of intervals between the injections. A total of approximately 3.0 mg of virus was distributed in the three injections. The immunized animal was bled every week, starting 10 days after the last injection. The antiserum was stored at -20°C by either freezing directly or after freezedrying.

For IgG purification, the antiserum was absorbed with extracts of health plants prepared in polyvinyl pirrolidone buffer pH 7.0. The antiserum was mixed with the extract, in the proportion of 1:10 (v/v) and the mixture was incubated at 37°C during 3h. The mixture was centrifuged at 10,000g for 10 min. The IgGs were purified from the absorbed antiserum by column chromatography with Protein A (7). The antiserum was initially filtered through a column of Protein A and the column was washed with the elution buffer pH 3.0 (0.1 M glycine HCl, 0.02% sodium azide and 0.1 mM phenylmethylsulfonyl-fluorid) to elute the IgGs from the Protein A gel. Aliquots of 1.5 ml of elution buffer were collected and analyzed in the UV spectrophotometer at 280 nm to detect the IgGs. The aliquots with absorption above 0.4 were mixed as they had IgGs in accepted concentrations. The final IgG concentration was adjusted to 1.2 to be used in enzyme linked-immunosorbent assay (ELISA).

Double immunodiffusion tests with the obtained antiserum were performed in agar medium containing 0.8% Noble agar; 0.5% SDS and 1.0% NaN₃ in distilled water. The antigens used as reactants were prepared in 1.5% SDS solution or in distilled water. The antigens and undiluted antiserum were pipetted directly into the appropriated wells punched in the solidified agar medium, and the plates were incubated in a moist chamber at 24°C for 24h.

The absorbed antiserum and the purified IgGs were tested by indirect ELISA. Leaf tissue was macerated in coating buffer at a 1:10 dilution (w/v) and used to coat microtiter plates which were incubated overnight at 4°C. The virus-specific IgGs were added at the follow dilution's: 1:100; 1:1,000 and 1:2,000. The plates were incubated at 37°C for 3 h and the goat anti-rabbit IgG alkaline phosphatase conjugate was added at a dilution 1:2,000 and the plates were incubated again at 37°C for 3 h. The reactions were measured at A₄₀₅nm absorbency on a MicroElisa Reader Labsystems Multiskan MS, 15, 30 and 45 min after addition of substrate. An ELISA reaction was considered positive if it exceeded two times the mean of the OD₄₀₅mm of healthy tissue controls and had a minimum reading of 0.2. In all the tests extracts from uninfected plants were included as control.

The antiserum was also tested by western blotting, using clarified infected and healthy plant extracts. The plant extracts
were submitted to SDS-PAGE. The proteins immobilized in the gel were electro transferred to a nitrocellulose membrane by the BRL-MINI-V8.10 system, using 20 mM Tris buffer, 150 mM glycine and 20% of methanol. The electrophoresis was performed at a temperature around 4°C and a constant voltage of 120V, during 2 h. The membrane was stained with the Ponceau-S reagent to permit the visualization of the protein bands. The Ponceau-S reagent was removed with distilled water and the membrane was treated with TBS containing 5% powder milk, during 45 min. After that the membrane was treated with the SMV antiserum or it purified IgGs diluted to 1:500 with TBS containing 1% of powder milk, during 45 min, after which the membrane was washed 5 times with TBS. The membrane was treated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma A-8025), at a dilution 1:2,500 in TBS containing 1% of powder milk. The reaction of the alkaline phosphatase with BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl phosphate/Nitro Blue Tetrazolium) in the reaction buffer containing 100mM Tris, 100 mM of NaCl and 5 mM of MgCl, pH 9.0, was used to visualize the proteins in the membrane.

3.6 - Seed Transmission Studies

Initially, a total of 70 seeds harvested from SMV infected soybean 'Bragg' plants grown at greenhouse conditions with temperature varying from 26°C (night) to 35°C (day) was tested for the presence of virus by the direct germination method in association with serology. The plants originated from those seeds were observed at greenhouse conditions during 30 days after planting for symptom development. All the plants showing symptoms and those with suspicion of symptoms were individually tested against SMV antiserum by double immunodiffusion method.

In a second experiment, seeds harvested from SMV systemically infected soybean plants 'Engopa' (41 seeds), 'IAC-2' (15), 'Pelicanos' (60), 'PI-240663' (35), 'Santa Rosa' (60) and 'Seridó' (30) were tested by ELISA at laboratory conditions. Seeds were surface-sterilized in 0.5% sodium hypochloride for 10 min, rinsed thoroughly with sterile distilled water and placed in moistened paper tower to germinate for 5 days at room temperature. Hypocotyls of germinated seeds were excised with sterile histoyre and used for testing in group of five against the purified IgG specific for SMV by ELISA. All the groups of five seeds that showed positive results in ELISA were retested individually to determine the percentage of infected seeds.

RESULTS AND DISCUSSION

The SMV isolate was capable to infect all the soybean cultivars inoculated, causing mosaic and leaf distortion in all of them, with the 'IAC-2' being the one of which showed the most severe symptoms and, consequently, demonstrated to be an excellent host for virus propagation and purification. Besides the soybean cultivar,
the time of virus inoculation, probably had a great influence in the virus replication. A good concentration of virus particles was observed 15 days after the 'IAC-2' cotyledonary leaves had been inoculated. Light microscopic observations of epidermal leaf strips prepared from systemically SMV infected 'IAC-2' plants revealed the presence of great number of cytoplasmic inclusions (Figure 1) similar to those reported for SMV (14), an indication of high virus concentration.

Some problems with the virus purification using the method described by Calvert & Ghabrial (8) were solved by modifications introduced in the clarification and initial precipitation of the virus particles. The leaf extracts were not treated

<table>
<thead>
<tr>
<th>Glycine max Cultivars</th>
<th>Tested Seeds</th>
<th>Seeds with SMV</th>
<th>% of transmission</th>
</tr>
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<tbody>
<tr>
<td>‘Engopa’</td>
<td>41</td>
<td>-</td>
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<tr>
<td>‘IAC-2’</td>
<td>15</td>
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<tr>
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<td>‘Santa Rosa’</td>
<td>60</td>
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<td>3.3</td>
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<tr>
<td>‘Serido’</td>
<td>30</td>
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Some problems with the virus purification using the method described by Calvert & Ghabrial (8) were solved by modifications introduced in the clarification and initial precipitation of the virus particles. The leaf extracts were not treated

Figure 2 - Photomicrograph of cytoplasmic inclusions in an epidermal strip prepared from leaf of soybean, Glycine max, 'IAC-2' systemically infected with soybean mosaic virus (SMV), stained with a combination of calcomine orange and luxol brilliant green. Cytoplasmic inclusions (CI), plant cell wall (CW) and nucleus (Nu).
virus preparations showed strong stream birefringence and the highest yield of the best virus preparation was estimated to be around 80 mg of virus per Kg of infected soybean leaf tissue. The purified SMV preparation was highly ineffective in soybean 'IAC-2', demonstrating that the purification process did not interfere with the biological integrity of the virus.

The PAGE analysis of a freshly purified preparation of SMV reveals a main protein component with an estimated molecular weight (MW) of 32k (Figure 4).

The purified virus preparation showed a UV absorption spectrum with a maximum at 260-261 nm and a minimum at 246-247 nm (Figure 3). The values obtained for the ratio of \( \lambda_{260}/\lambda_{280} \) was approximately 1.2 and for the ratio \( \lambda_{\text{max}}/\lambda_{\text{min}} \) was 1.09. The purified

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**Figure 3 - Ultraviolet absorption spectrum of purified preparation of the isolate of soybean mosaic virus (SMV) obtained from seeds of Glycine max 'Bragg'.**

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**Figure 4 - Polyacrilamide gel electrophoresis of SDS-dissociated viral coat protein of freshly purified soybean mosaic virus (SMV). Photograph of gel showing the virus coat protein component and its molecular weight (arrow) estimated on the basis of the migrations of the following marker proteins: a) bovine serum albumin (66k), b) ovalbumin (45k), c) pepsin (34.7k), d) trypsinogen (24k), e) and B-lactoglobulin (18.4k).**
the best approach currently available to minimize the losses caused by the virus (20). The fact that the virus was not transmitted by seeds of 'Engopa', 'IAC-2', and 'Serido', and presented very low rate of seed transmission with other cultivars used for production of virus infected seed at greenhouse conditions (Table 1) could be explained by the variability among the soybean cultivars tested and the high temperature in which the seeds were produced. According to Goodman et al. (16) some soybean cultivars do not support SMV transmission through the seeds. On the other hand, the day temperature in the greenhouse varied from 27 to 34°C. The 70% of virus transmission through the 'Bragg' seeds could be explained by the fact that those seeds were produced in Paraná and the SMV is normally transmitted in high rate by seeds of this soybean cultivar.

The obtained polyclonal antiserum was reactive in SDS-immunodiffusion and in-

![Figure 5 - Double immunodiffusion test with antiserum specific for the isolate of soybean mosaic virus (SMV) obtained from seeds of Glycine max 'Bragg'. A-prepared antiserum; V-extracts from SMV-infected plants, and H-extracts from health plants. A - prepared antiserum; V - extracts from SMV infected plants and H - extracts from health plants.](image)

![Figure 6 - Enzyme linked immunosorbent assay (ELISA) with purified IgGs from the antiserum prepared to the isolated of soybean mosaic virus (SMV) obtained from infected seeds of Glycine max 'Bragg', using different dilutions.](image)
direct-ELISA tests with saps of infected plants without showing any reaction with extracts of non inoculated plants in double diffusion (Figure 5). The purified IgG showed a good concentration and was also very specific to SMV in indirect ELISA tests, with positive results up to a dilution of 1:2,000 (Figure 6). The western blotting results also confirmed the antiserum specificity. The nitrocellulose membrane treated with the SMV antiserum showed reactions with the protein bands from the purified virus preparation and the infected plants extracts, corresponding to a molecular weight equivalent to the capsidial virus protein.

This seems to be the first specific antiserum produced for SMV in Brazil. Since it showed to be very specific and useful for different serological tests, including to detect the presence of the virus in infected seeds and determine the rate of seed transmission by ELISA, it will be of great value for diagnose and research purposes.

REFERENCES


soybean cultivars. Phytopathology 69: 467-470.


