

Biological and Physicochemical Properties of Cowpea Severe Mosaic Comovirus Isolated from Soybean in the State of Paraná

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ABSTRACT

Soybean plants with symptoms of bud blight were growing close to cowpea with severe symptoms of mosaic associated with blisters in the leaves. A group of plants of both species were collected and used for etiological studies. This kind of symptom in soybeans was common in certain areas of the State of Paraná, induced by tobacco streak ilarvirus. Host range, serological reaction, particle morphology and size, protein and nucleic acid analysis, and transmission by beetles from species *Cerotoma arcuata* Oliv. showed that the virus involved was cowpea severe mosaic comovirus. This is the first report on the occurrence of this virus in soybean plants in the State of Paraná. Results using indirect ELISA showed that in cowpea the relative virus concentration was higher in green leaf areas than in chlorotic ones. Also, virus concentration, determined through indirect ELISA was much higher in plants kept at diurnal regime of 25°C x 23°C (12 x 12 h) than at 30°C x 28°C.

Key words: soybean; cowpea severe mosaic comovirus; Indirect ELISA

INTRODUCTION

Soybean is the most important grain crop in Brazil and occupies an area of 12 million hectares. The yield average has increased along the years to 3,000 kg/ha (Roessing & Guedes, 1993). Despite the high yields, it is not unusual to find farmers claiming for losses caused by diseases such as soybean cyst nematode and stem canker (Mendes & Dickson, 1992; Wrather et al., 1997).

Viruses have been also reported to cause damage to soybeans, and at least 13 viruses have been described naturally infecting soybean in the fields (Costa, 1977; Almeida, 1994). A symptom observed in soybean fields, called bud blight could be induced by several viruses (Costa, 1988). However, in the State of Paraná this symptom has been associated with the infection caused by tobacco streak ilarvirus (Costa & Carvalho, 1961) and was responsible for severe losses to the farmers in the past (Almeida et al., 1995).

In Londrina, State of Paraná, soybean plants

with bud blight were observed close to cowpea plants exhibiting severe mosaic and leaf distortion. Samples from both species were collected for etiological studies and the results showed that the same virus was responsible for the symptoms in both plants. The virus was identified as cowpea severe mosaic comovirus (CpSMV) with characteristics similar to those already described (Anjos & Lin, 1980). CpSMV was first reported in Brazil (Oliveira, 1947). Later, the virus was also mentioned in Northeast and Central regions of Brazil (Costa et al., 1969; Lima & Nelson, 1977; Cupertino et al., 1981; Rios & Neves, 1982). It has three separate components in sucrose gradient, with 58, 98 and 118 S values for the top, middle and bottom component, respectively. It has a bipartite genome and is transmitted by beetles from the genus *Cerotoma* (Jager, 1979). Serological evaluations of CpSMV isolates from different Brazilian regions have shown the occurrence of four serotypes (Lin et al., 1980).

This study was done to identify and characterize the isolate of CpSMV as well as to evaluate its

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biological and physicochemical properties. Additionally it was evaluated the relative virus concentration between green and chlorotic leaf areas as well as the virus concentration in cowpea plants kept at two different regimes of temperature, trying to simulate temperature effects, commonly found in Northern and Southern regions of Brazil.

MATERIAL AND METHODS

Virus isolation and maintenance. Soybean and cowpea leaves showing symptoms of systemic necrosis and severe mosaic, respectively, were collected from the field and used as source of inoculum. Leaves were ground in a sterile frozen mortar with sodium phosphate buffer 0.01M, pH 7.0 (1:5 w/v). The extract was applied to the soybean and cowpea leaves previously dusted with charcoal. Inoculated leaves were lightly washed and the plants were kept in greenhouse.

Transmission trials. Aphids (*Myzus persicae* Sulz.) were reared in raddish (*Raphanus sativus* L.). After starvation for 1h, insects were transferred to systemically infected leaves of cowpea and allowed to probe for 5 min. Five aphids per plant were transferred to healthy soybean cv. Santa Rosa and cowpea cv. Blackeye where they stayed for 24 h. Plants were then sprayed with malathion. Beetles (*Cerotoma arcuata* Oliv.) raised on healthy french beans (*Phaseolus vulgaris* L.) cv. Tibagi, were transferred to cowpea plants systemically infected and allowed to feed for 72 h. Insects were then transferred to soybean and cowpea plants, kept inside cages for 72 h and killed with malathion. Five insects per plant were used. Plants were scored for symptoms 2-3 weeks later.

Electron microscopy. Virus particles were visualized through a leaf dip method (Kitajima, 1965). Additionally, purified preparation was observed after negative staining using 2% uranyl acetate.

Purification. Cowpea leaves cv. Blackeye, systemically infected were harvested

approximately three weeks after inoculation and ground in blender with sodium phosphate buffer 0.1 M, pH 7.2, 0.01 M EDTA and sodium sulfite 1% (1:2 w/v). The emulsion was mixed with butanol (8%), agitated for 20 min at 4°C and stored for another 10 min. The slurry was centrifuged at 10,000 x g for 10 min. Polyethylene glycol (molecular weight 6,000) was added to the supernatant to a final concentration of 8%, stirred for 1 h at 4°C. Precipitate was recovered after centrifugation at 12,000 x g for 10 min and resuspended in 1/10 initial volume with 0.1 M sodium phosphate buffer, pH 7.2, stirring overnight at 4°C. Solution was clarified by centrifugation at 10,000 x g for 10 minutes and the supernatant was centrifuged at 154,000 x g for 2 h. Pellets were resuspended in a total volume of 2.4 ml sodium phosphate buffer 0.01 M, pH 7.2. Virus preparation was layered on frozen solution of sucrose (20%) (Baxter-Gabbard, 1972; Davis & Pearson, 1978) and centrifuged at 134,000 x g for 2 h. Tubes were scanned in ISCO fractionator, model UA-5, monitoring UV absorbance at 254 nm. Peaks were collected, diluted 3x with sodium phosphate buffer 0.01 M, pH 7.2 and again centrifuged at 134,000 x g for 2.5 h. Pellets were finally resuspended in 0.3 ml of the same buffer and subjected to 8,000 x g for 10 min. Supernatant was used for spectrophotometry.

Protein analysis. The molecular weight of viral coat protein was estimated by electrophoresis in sodium dodecyl sulfate (SDS) polycarilamide gels consisting of a 3.75% stacking gel and 12.5% resolving gel (Laemmli, 1970). Protein was dissociated from its RNA by boiling the virions for 3 min in 0.125 M Tris - HCl buffer, pH 8.3, containing 1% SDS, 1% 2-mercaptoethanol and 4 M urea. Mobility of the protein molecular markers (Pharmacia LMW calibration kit) were used for estimating the viral protein molecular weight. Gels were stained with coomassie brilliant blue R250 (Sigma, St. Louis, USA).

Serology. Antiserum was prepared using New Zealand rabbits in which it was given three intramuscular injections at a week's interval, receiving a total of 3 mg of virus. Bleeding was carried out at 10 days interval after the final

injection. The first injection was prepared with complete Freund's adjuvant and the second and third were prepared by using incomplete adjuvant. Other antisera against CpSMV were provided by Dr. J.A.A.Lima (Universidade Federal do Ceará) and Dr. P.S.T.Brioso (Universidade Federal Rural do Rio de Janeiro). Double diffusion tests were performed in 0.8% agarose containing 0.02% sodium azide in distilled water by using patterns of six wells around a central well. Evaluations were recorded 24 h after incubation of the plates in humid chamber at room temperature.

Host range tests. At least eight plants from different botanical families were mechanically inoculated as described before using inoculum prepared from cowpea leaves systemically infected, homogenized in 0.01 M sodium phosphate buffer, pH 7.0. Symptoms were recorded 2-3 weeks after inoculation.

Virus content. The relative virus content was measured three weeks after inoculation by indirect ELISA (Koenig, 1981) using samples from green and chlorotic areas in cowpea leaves showing symptoms of mosaic. The experimental design for this experiment was completely randomized with three replications. Each pot containing three plants was considered one replication. Three weeks after inoculation the primary leaf and the middle leaflet from the second, third, fourth and fifth trifoliolate leaves of each plant were collected. Leaves from the same position and from plants in the same pot were stacked and cut with a cork borer (3.4 cm²). Sample disks were ground on coating buffer (pH 9.6) (1:2 w/v), diluted and directly coated into the microtitre plates (100 µl/well) overnight at 4°C. Optimum working dilution was 1:300 for the rabbit IgG and 1:50 for the plant extract. Alkaline phosphatase conjugate to goat and anti-rabbit IgG (Sigma, USA) was diluted 3,000-fold. Absorbance was recorded at 405 nm, 30 min after adding the substrate p-nitrophenyl phosphate (Sigma, USA). The reaction was stopped with 3N NaOH. Mean absorbances were determined from triplicate wells. Between each incubation step, wells were washed with PBS. A sample was considered positive only when it had two times the absorbance values of the control.

Temperature effect on virus content.

Mechanically infected plants of cowpea cv. Pitiúba were submitted to two different regimes of temperature (25°C x 23°C; 30°C x 28°C) in 12 x 12 h of light and darkness, respectively. At 7, 15, 22, 29 and 35 days after inoculation (DAI), five plants of each treatment were collected and the leaves used for indexing relative virus content by indirect ELISA.

RESULTS AND DISCUSSION

After mechanical inoculation the virus was able to infect several plant species (Table 1). All soybean cultivars tested showed severe necrosis and bud blight 10-12 DAI. Cowpea plants cv. Pitiúba showed severe mosaic associated to blisters while cv. Blackeye exhibited systemic necrosis. *Chenopodium amaranticolor* and *C. quinoa* exhibited small, circular, chlorotic local lesions about eight days after inoculation. These lesions later became necrotic. The virus was not transmitted through *Myzus persicae* Sulz. but it was through *Ceratomyza arcuata* Oliv. Seven plants out of ten showed infection.

Following sucrose density gradient centrifugation, purified preparation showed three ultraviolet absorbing bands (Fig. 1). Average yields of purified virus ranged from 5-9 mg/100g of infected tissue, calculated from extinction coefficient of 10.0 (Van Kammen, 1971). Middle and bottom components were observed in large amounts. Purified virus showed an ultraviolet absorption spectrum typical of nucleoprotein with maximum absorption at 260-262 nm and minimum at 240-242 nm. The A_{260}/A_{280} ratio for unfractionated virus was in the range of 1.50 and 1.68 with a mean of 1.53 for six preparations (values not corrected for light scattering). Analysis of CpSMV denatured proteins resolved as two separate bands under reducing conditions showing a molecular weight of 42.1 KDa and 18.2 KDa (Fig 1C) which are in accordance to

Table I. Reaction of several botanical species mechanically infected by cowpea severe mosaic comovirus.

Species	Reaction
Fam. Leguminosae	
<i>Glycine max</i> (L.) Merr.	
cv. Davis	M/E
cv. Santa Rosa*	SM
<i>Phaseolus vulgaris</i> L.	
cv. Carioca*	NS
cv. Jalo*	SM/Y
cv. Manteiga*	M/CLL
cv. Rosinha*	M/CLL
cv. Tibagi*	NS
<i>Vigna unguiculata</i> (L.) Walp.	
Cv. Black eye	SM/CLL/SN
<i>Crotalaria spectabilis</i> Roth.	M/CLL
<i>Crotalaria juncea</i> L.	M/CLL
<i>Crotalaria striata</i> Dc.	NS
<i>Crotalaria mucronata</i> Desv.	NS
Fam. Solanaceae	
<i>Lycopersicon esculentum</i> Mill.	NS
<i>Datura stramonium</i> L.	NS
<i>Nicotiana tabacum</i> L.	NS
Fam. Chenopodiaceae	
<i>Chenopodium amaranticolor</i> Coste & Reyn	CLL/NLL
<i>Chenopodium quinoa</i> Willd	CCL
<i>Chenopodium murale</i> L.	LLC
Fam. Compositae	
<i>Bidens pilosa</i> L.	NS
<i>Gomphrena globosa</i>	M/CLL
Fam. Cucurbitaceae	
<i>Cucurbita pepo</i> L.	NS

NS: no symptoms; M: mosaic; SM: severe mosaic; CLL: chlorotic local lesion; NLL: necrotic local lesion; Y: yellowing; E: epinasty; SN: systemic necrosis.

* Plants indexed in *C. amaranticolor*.

previous reports (Jager, 1979; Brioso et al., 1996). Good agreements were observed between various estimates which suggested that the protein did not behave anomalously and bound the usual 1.4 g of SDS/g of protein (Pitt-Rivers & Impiombato, 1969). In the electron microscope, viral preparation contained isometric particles measuring 29 nm in diameter (Fig. 1B).

Sap from infected cowpea reacted strongly with antisera of CpSMV-Pi, CpSMV-Ce, and

CpSMV-RJ. Antiserum produced against this isolate showed a titer of 1/512 and reacted with healthy tissue at a titer less than 1/8 dilution.

The effect of temperature on plant virus replication or translocation has been mentioned by Dawson et al. (1975). Data shown in this paper confirmed those effects but in a different order. Instead of an increase in relative virus concentration a decrease was detected, meaning that at low temperatures the virus

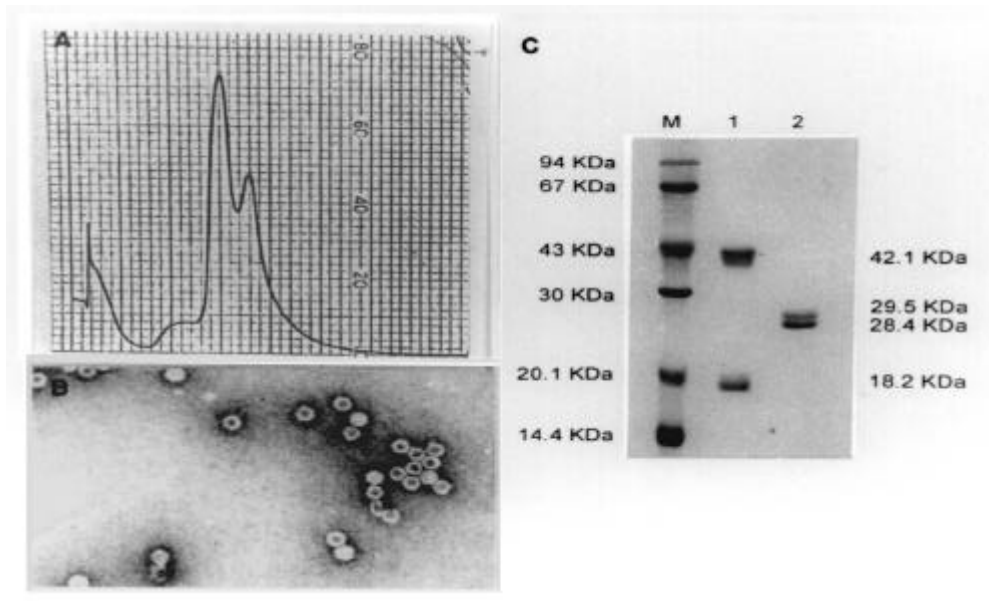


Figure 1. (A)Ultraviolet absorbance profiles of cowpea severe mosaic virus purified from soybean by rate zonal sucrose gradient (20% frozen sucrose); (B)Electron microscopy of CpSMV showing particles 29 nm diameter; (C)Molecular weight of the capsid protein from cowpea severe mosaic virus; Lane M (markers): Fosforilase b, 94 Kda; bovine serum albumin 67 KDa; egg albumin, 43 KDa; carbonic anhydrase, 30 KDa; trypsinogen, 20 Kda and lactalbumin, 14.4 KDa; Lane 1 = cowpea severe mosaic virus coat protein; Lane 2 = Tobacco streak virus coat protein.

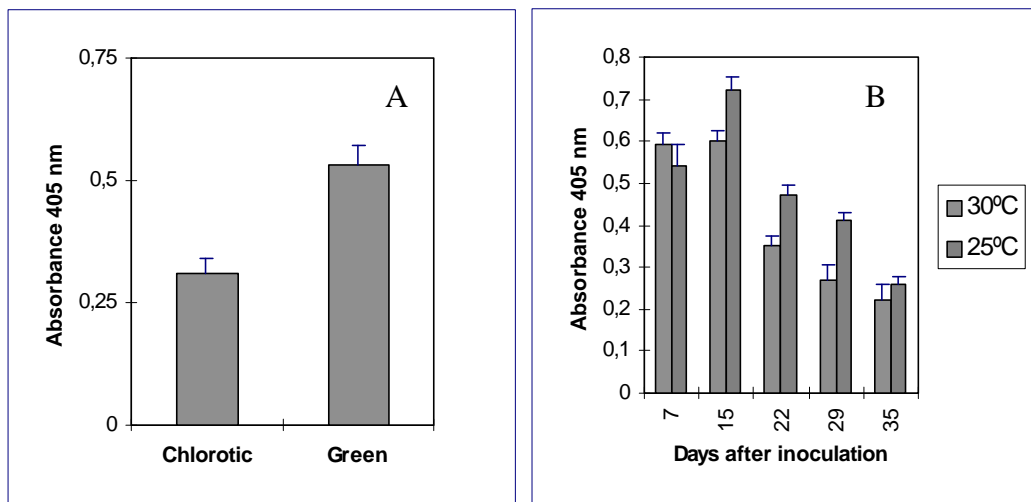


Figure 2. A: Relative content of cowpea severe mosaic virus in leaf samples from chlorotic and green areas, measured by indirect ELISA. Error bars correspond to standard error of the means; B: Relative content of cowpea severe mosaic virus, determined by indirect ELISA in cowpea leaves at 7, 15, 22, 29 and 35 days after mechanical inoculation. Plants were maintained at two regimes of temperature (30x28°C e 25x23°C). Error bars correspond to standard error of the means.

replicated better than at 30°C. Probably 30°C was not the ideal temperature for replication of this isolate. Virus concentration tended also to decrease along the days after inoculation. It was not clear if this fact was only due to the chlorosis and systemic necrosis symptoms observed in cowpea leaves or was due to any other fact not identified in this work. Although evaluating biochemical or physiological differences between green and chlorotic areas was not an objective previously targeted for this study, it was interesting to observe that chlorotic areas had less virus, suggesting that an interference could be associated with this event. An additional cytological study may confirm viral effect on cell organelles that could explain this finding.

Soybean bud blight induced by tobacco streak virus was a symptom usually found in the past among soybean fields located at Southern States of Brazil (Costa, 1977; Almeida et al., 1995). Several viruses can infect soybean and induce symptoms of bud blight (Costa, 1988). One of them is cowpea severe mosaic virus (CpSMV). The occurrence of CpSMV naturally infecting soybeans in Paraná is a new event. This symptom was first described in soybean fields in Brasília, Federal District (Anjos and Lin, 1980). Results obtained in this study such as particle size and morphology, coat protein size, nucleic acid profile, particle sedimentation profile in sucrose gradient, host range and serological relationships confirmed the natural occurrence of CpSMV in soybeans, grown in the State of Paraná. A previous report of this study showed that this isolate was serologically identical to others identified in Northern states (Bertacini et al., 1994).

Because of the severity of the disease in soybeans, this virus poses a serious threat to this crop in traditional areas, as well as to new areas in Northern States where soybean is expanding steadily. So far, no genetical source of resistance has been identified in the soybean germplasm bank of Embrapa Soja (Almeida, 1996). Cowpea is a traditional and important source of protein for human nutrition in Northeastern States, where genetical resistance against CpSMV has been used efficiently. However, the introduction

of soybeans in that area may be threatened by this virus. Moreover, very few epidemiological parameters associated to virus spreading and persistence in the field are well understood in this crop.

This paper describes etiological studies related to the occurrence of soybean bud blight caused by CpSMV and constitutes the first occurrence of this disease in soybeans grown in the State of Paraná.

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