Detection and Partial Characterization of a Carlavirus Causing Stem Necrosis of Soybean in Brazil

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ABSTRACT

Stunting and stem necrosis were noticed in soybeans (*Glycine max*) grown in 2000/2001 in West Central Brazil the same condition was also observed in the following year in plantations as far as 2,000 km from the initial area. Based on transmission (mechanical, graft, insect vector), purification and serology, electron microscopy and molecular studies the causal agent was determined to be a whitefly-borne carlavirus which is possibly related to *Cowpea mild mottle virus* (CpMMV).

Additional keywords: *Cowpea mild mottle virus,* whitefly, *Bemisia tabaci* biotype B.

RESUMO

Detecção e caracterização parcial de um carlavirus causando necrose da haste da soja, no Brasil

No ano agrícola de 2000/2001, ocorreu um surto de nanismo e necrose da haste em soja (*Glycine max*) plantada em duas áreas do Brasil Central e na safra seguinte, esta anomalia foi constatada em outras regiões produtoras, mesmo distantes mais de 2.000 km de onde fora inicialmente constatada. Estudos envolvendo ensaios de transmissão (enxertia, mecânica e insetos vetores), microscopia eletrônica, purificação, sorologia e ensaios moleculares indicaram que a enfermidade foi causada por um carlavirus transmitido por mosca branca, possivelmente relacionado ao *Cowpea mild mottle virus* (CpMMV).

Palavras-chave adicionais: *Cowpea mild mottle virus,* mosca-branca, *Bemisia tabaci* biotipo B.

Brazil is the world's second largest soybean [*Glycine max* (L.) Merrill] grower, producing 47 million tons over a cultivated area of 21 million ha for the year 2002 (CONAB, 2005). Soybean fields are spread throughout most of the country ranging from 31° latitude South to 4° latitude North. Most of the pathogens that infect soybean are caused by different species of fungi (Almeida *et al.*, 1997). In the 2000/01 season, soybean plants with symptoms of dwarfing and stem necrosis were observed in large areas of West Central Brazil (Goiatuba and Morrinhos, State of Goiás). Due to severe stem necrosis and death of the plants, the symptoms were initially thought to be caused by a fungal infection. However, all efforts for isolation and identification of the pathogen from roots of infected plants and necrotic stems were unsuccessful. A new outbreak occurred again in 2001, this time devastating soybean fields in different regions (Acreúna, Quirinópolis, Porteirão and Luiziana, State of Goiás; Sorriso, State of Mato Grosso; Barreiras, State of Bahia; Balsas, State of Maranhão) some of them as far as 2,000 km from the previous occurrence. Recently, the disease was identified in Palotina, State of Paraná. Samples from infected plants were grafted on plants from the soybean cultivar Embrapa 63. Symptoms of mosaic, dwarfing and bud blight developed two-three weeks later, indicating a probable viral etiology. Stem necrosis is not a common virus symptom, but bud blight has been associated, in Brazil and Argentina, with at least three viruses (Costa, 1977; Anjos & Lin, 1984; Laguna *et al.*, 1988; Almeida, 1994). In this paper we describe the first report of a whitefly-borne virus causing dwarfing and stem necrosis in soybeans in Brazil.

Buds and stems from infected soybean plants were collected from the field, at Barreiras, in the State of Bahia, and grafted on soybean cvs. Embrapa 63 and CD-206. Plants were covered with plastic bags for four days and kept in a greenhouse at an average temperature of 27 °C. Infected leaves with symptoms of mosaic were also used for mechanical inoculation. One gram of leaf tissue was ground in 5 ml of 0.01 M sodium phosphate buffer pH 7. The extract was rubbed onto leaves of several botanical species, previously dusted with fine charcoal and later washed with water. Symptoms were evaluated at seven-day intervals after inoculation.

Nonviruliferous aphids, *Myzus persicae* (Sulzer) and *Uroleucon ambrosiae* (Thomas), were maintained in turnip (*Brassica rapa* L.) and *Bidens pilosa* L., respectively. After starving for 1 min, insects were transferred to infected soybean leaf for 5 min, 15 min, 30 min and 24 h and then five insects were transferred to each healthy soybean plant of cvs. Embrapa

63 and CD-206. After an inoculation period of 3 h, an insecticide (monocrotophos) was used to kill the insects. Whiteflies (*Bemisia tabaci* (Gennadius) biotype B (= *B. argentifolii* Bellows & Perring)) were maintained in soybean and given an access period of 24 h on infected soybean plant. Ten insects were transferred to a healthy plant and left to feed for 48 h, then killed by spraying an insecticide (0.5% monocrotophos). In both tests evaluations were done at seven-day intervals. For tests of seed transmission soybean plants cvs. Embrapa 63 and CD 206 were infected at the second trifoliolate leaf stage and 2,000 seeds were sown for transmission tests.

Symptomatic leaves of common bean (*Phaseolus vulgaris* L.) cv. Jalo and soybean cv. Embrapa 63 were used for virus purification according to Gaspar & Costa (1993). The molecular weight of the capsid protein was estimated by sodium dodecyl sulfate (SDS) – polyacrilamide gel electrophoresis (SDS-PAGE) on a 3.5% stacking gel and a 12% resolving gel. After electrophoresis for 2 h at 120 V at room temperature, proteins were stained with coomassie brilliant blue. Protein molecular weight was estimated by comparing their relative mobility with molecular weight standards (Gibco BRL).

Immunosorbent electron microscopy (ISEM) was performed with grids coated with Butvar and carbon film. They were floated on drops of *Cowpea mild mottle virus* (CpMMV), family *Flexiviridae*, genus *Carlavirus*, antiserum diluted 1:1000 in 0,05 M phosphate buffer pH 6.5 for 30 min at room temperature and then washed with five drops of the same buffer. Later, they were drained with filter paper and transferred to drops of antigen (extract of soybean-infected leaves) for 30 min at room temperature. Afterwards they were washed consecutively with five drops of phosphate buffer and five drops of distilled water and negatively stained with uranyl acetate 2%, and examined in a Zeiss EM-10 electron microscope. To "decorate" the virus particles, after the grids had been transfered to drops of antigen, they were floated on drops of the same antiserum for 1 h at room temperature and then passed through the other procedures.

Electron microscopy was also performed with infected leaves that were cut into small pieces and fixed in a modified Karnovsky fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2), post-fixed with 1% OsO_4, dehydrated in acetone and embedded in Spurr´s low viscosity resin. Sections were stained with uranyl acetate and Reynold´s lead citrate and examined in a Zeiss EM 900 transmission electron microscope. Attempts to observe presumed viral particles were also made in leaf dip preparations negatively stained with sodium silicotungstate (Kitajima & Nome, 1999).

Total RNA was extracted from 100 mg of fresh leaf tissue ground in liquid nitrogen according to the RNeasy Plant Kit protocol (Qiagen). The cDNA was synthesized using reverse transcriptase (Invitrogen) and buffers according to the manufacturer in a final volume of 30 µl. From the total RNA extraction solution, 10 µl were taken and mixed with 2 µl of primer oligo-dT_{21} (10 µM) (Badge *et al*., 1996), incubated at 80 °C for 3 min and quenched on ice for at least 1 min. After that, other reaction components were added in the following order: 6 µl of 5X RT (reverse transcriptase) first strand buffer (200 mM Tris-HCl, pH 8.4), 1 µl of dNTP mix (10 mM), 2 µl of DTT (0.1M), 2 µl M-MLV reverse transcriptase (20 U) (Invitrogen), and DEPC-treated water for a final reaction volume of 30 µl. The tubes were incubated for 1 h at 37 °C on a Perkin Elmer model 9600 thermocycler followed by 10 min of heating at 65 °C to denature the enzyme. The PCR reactions were performed in a 30 µl volume containing 4 µl of PCR buffer (10X), 3 µl of $MgCl_2$, 1 µl of dNTP mix (10 mM), 1 µl of Taq DNA polimerase (5U/µl), 4 µl of cDNA, 23 µl of DEPC-treated water and 2 µl of each primer, oligo-dT_{21} and Carla-Uni (5´-GGAGTAACCGAGGTGAT ACC-3´) (Badge *et al.*, 1996). The reaction mixture was heated at 94 °C for 3 min and then submitted to 35 cycles of 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 2 min, followed by 10 min incubation at 72 °C.

Both strands were sequenced by the dideoxy chain-termination method, using a 377A DNA sequencer (Applied Biosystems). Nucleotide sequences were analyzed and compared with sequences deposited at the GenBank and EMBL databases. Multiple sequence alignments were produced using CLUSTAL W.

Virus was sap-transmitted to soybean plants reproducing the symptoms of bud blight, dwarfing and mosaic normally associated with blisters. Stem necrosis did not occur as frequently as in the field. Four out of the 25 plant species tested were infected by the viral isolate studied, indicating a narrow host range (Table 1). The virus isolate was able to systemically infect peanut (*Arachis hypogaea* L.), common bean (cv. Jalo and Tibagi) and three soybean (*Glycine max* L.) cultivars, and caused necrotic local lesions in *Chenopodium amaranticolor* Coste & Reyn. The virus was not transmitted by aphids, but it was transmitted by whiteflies with an average efficiency of 65%. From a total of 2,000 seeds harvested from previously infected plants, 1,885 seedlings were obtained and evaluated up to 60 days after emergence. Not a single plant developed symptoms of mosaic, bud blight or stem necrosis.

The method used for purification yielded an average of 14 mg of virus / 100 g soybean leaf tissue. SDS-PAGE analysis of purified preparations indicated the presence of a single protein with a relative molecular mass of 30.12 ± 1.85 kDa (mean of three determinations ± standard error). This is consistent with values reported for plant viruses from the genus *Carlavirus* (Brunt *et al*., 1996).

Flexuous particles *ca*. 15 nm x 600-700 nm were consistently detected in leaf-dip preparations from infected plants either naturally or experimentally infected (Figure 1A). Serological tests performed through immunosorbent electron microscopy (ISEM) showed a highly positive decoration results obtained with the CpMMV antiserum (Figure 1B). The cytopathology of the tissues infected by stem necrosis virus (Figure 1C) was characterized by the presence, in the cytoplasm, of feather-like inclusions formed by presumed virions, similar to that described for CpMMV (Brunt *et al*., 1983; Gaspar & Costa, 1993).

The short nucleotide sequence obtained showed significant similarity (84.8%) to the CpMMV sequence

TABLE 1 - Indicator plants and reactions after sap inoculation with the virus isolated from soybean (*Glycine max*) plants

Family	Species	Symptom reaction to the virus
Leguminosae	*Glycine max* L. Merr	
	cv. Santa Rosa	-
	cv. Embrapa 63	SN/Mo
	cv. CD 206	Mo
	cv.BRS 133	-
	cv. UFV 19	SN
	Phaseolus vulgaris L.	
	cv. Carioca	-
	cv. Jalo	M/C
	cv. Tibagi	Mo
	Arachis hipogea L.	Mo
	Cajanus cajan (L.) Millsp.	-
Cucurbitaceae	*Cucurbita pepo* L. cv. Caserta	-
Compositae	*Bidens pilosa* L.	-
Labiatae	*Ocimun basilicum* L. Sprengel	-
Gramineae	*Zea mays* L.	-
Pedaliaceae	*Sesamun indicum* L.	-
Amaranthaceae	*Amaranthus hybridus var. paniculatus* (L.) Thell	-
	Gomphrena globosa L.	-
Chenopodiaceae	*Chenopodium amaranticolor* Coste & Reyn.	NLL
	Chenopodium quinoa Willd	-
	Chenopodium murale L.	-
	Crotalaria mucronata Desv.	-
	C. pallida Aitona	-
	C. spectabilis Roth	-
	Vigna unguiculata (L.) Wallp. cv. Blackeye	-
Compositae	*Helianthus annuus anuus* L.	-
	Emilia sonchifolia (L.) DC.	-
Solanaceae	*Lycopersicon esculentum* .Mill	-
	Nicotiana tabacum L. Sansun NN	-
	N. glutinosa L.	-
	N. benthamiana Domin.	-
	Datura stramonium L.	-

*SN= systemic necrosis; Mo= mottling; M=mosaic; C= chlorosis; NLL= necrotic local lesion; -= no symptom and no virus detected after indexation on *C. amaranticolor*.

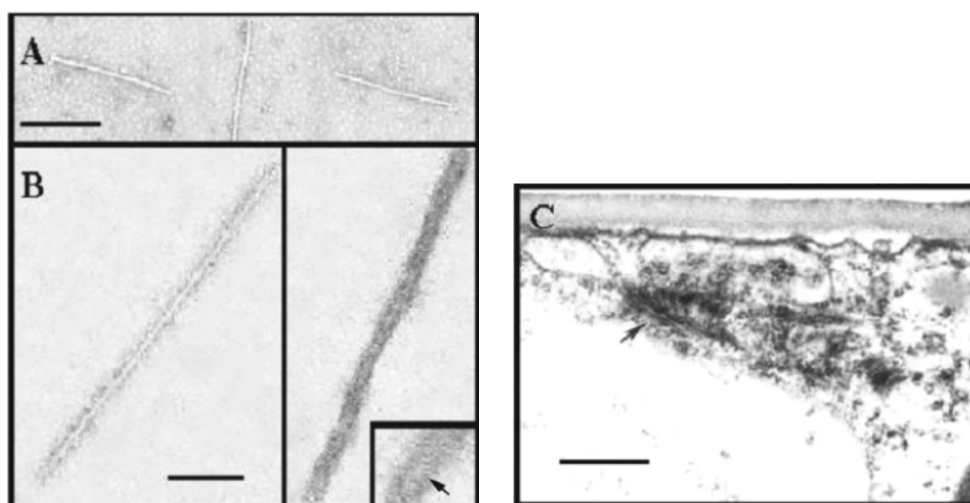

FIG. 1 - (A) Leaf-dip preparation from a soybean (*Glycine max*)-infected leaf. Bar = 500 nm. (B) Decoration test of the soybean virus particles. Left, untreated control; Right, treated with CpMMV antiserum. Inset:Note the antibody molecules attached to the soybean virus particle (arrow). Bar = 120 nm. (C) Transmission electron micrograph of virus particle aggregates (arrow) in the cytoplasm. Bar: 500 nm.

5´-GAGTAACCGAGGTGATACCCCTCATTTAAGGGAAAAAAACTCTTGAACCAAGGAAAGAGTA
TAAAGAGTCCTTGTGCATCTTCAGACCTAACTGTTGAGATGAAAACCGGTTTTAAAGTTATTTT
CCTGG3'-3´

FIG. 2 - Nucleotide sequence of the soybean (*Glycine max*) virus-fragment obtained by PCR with Carla-Uni and oligo $d(T_{21})$ primers.

(GenBank AF 024629) (Figura 2).

All the available data indicate that this soybean disease characterized by stunting and stem necrosis is caused by a whitefly-borne carlavirus. The initial suspicion of the involvement of a carlavirus, based on the finding of carlavirus-like particles and cytopathic effects in field soybean plants showing stem necrosis and stunting by electron microscopy, was later confirmed by transmission, purification and molecular analyses. Its identity has yet to be confirmed, but the 84% sequence similarity of a PCR-amplified small segment of 130 bp with CPMMV, another leguminous whitefly-borne virus (Brunt and Kenten, 1973; Iwaki *et al.,* 1982), suggests that it is either an isolate of this species, or a new, closely related species. The narrow host range observed agrees well with what has been reported for CpMMV in Ghana, Africa. However, CpMMV is reported to be seed borne (Brunt and Kenten, 1973), but the soybean virus was not transmitted in this manner. CpMMV has already been identified in Brazil causing mosaic in bean cv. 'Jalo' (Costa *et al.*, 1983; Gaspar *et al.,* 1985).

Bemisia tabaci was cited as a vector of CpMMV by Iwaki *et al.* (1982) and Costa *et al.* (1983). Later, Thouvenel *et al.* (1982) demonstrated that the virus was non-persistently transmitted. This is the first report of the transmission of a carlavirus by *B. tabaci* biotype B.

The fact that at least two soybean cultivars were not infected by this carlavirus indicates that they may serve for future breeding programs for resistance against the virus. For the time being, considering its possible relationship with CpMMV, it is suggested that this soybean carlavirus be referred to as a soybean isolate of CpMMV (CpMMV-S). This is the first report of a carlavirus naturally infecting soybean in Brazil.

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