Soybean blotchy mosaic virus, a new *Cytorhabdovirus* found in South Africa

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Abstract

A previously unidentified plant rhabdovirus associated with a blotchy mosaic symptom of soybean (*Glycine max*), prevalent in the lower lying, warmer soybean production areas of South Africa, was isolated and partially characterized. The virus was shown to be transmitted by mechanical inoculation and at least one species of leafhopper (*Peragallia caboverdensis* Lindberg (Cicadellidae, Agalliinae)). To determine the morphology and virion size, as well as intercellular accumulation, negative-stained preparations or embedded ultra-thin sections of infected plant samples were observed under a transmission electron microscope. The distribution of the virions within the cytoplasm, its bullet-shaped morphology and size (338-371nm x

93nm) suggested that it is a putative member of the genus *Cytorhabdovirus*. Degenerate primers designed to a conserved region of the polymerase gene of a number of rhabdoviruses were used in reverse-transcriptase polymerase chain reaction (RT-PCR) with total RNA from symptomatic plants as template. Amplicons were sequenced and compared to related sequences available on Genbank. The analysis confirmed that the virus was related to cytorhabdoviruses, with the highest nucleotide similarity being 60.7% with *Northern cereal mosaic virus*. The particle morphology, typical virion accumulation in the cytoplasm of infected cells, nucleotide sequence similarity with that of other plant rhabdoviruses, and unique symptoms on soybeans, suggest that the virus is a previously unknown cytorhabdovirus, for which we propose the name soybean blotchy mosaic virus (SbBMV).

Additional keywords: *Cytorhabdovirus*, phylogenetics, polymerase gene, *Rhabdoviridae,* leafhopper vector, *Peragallia caboverdensis*.

Introduction

During a survey of the viruses of soybean (*Glycine max* Merr.), of South Africa conducted from 1993 to 1995, an unknown virus-like disease was commonly found in the Brits/Thabazimbi soybean production area of the Northwest Province and Groblersdal/Loskopdam area of Mpumalanga (23), amongst the main soybean production areas of South Africa. Typically affected soybean plants displayed distinct blotchy mosaic symptoms (Figure 1) on the leaves. Preliminary morphological characterization of the virus had suggested that it was a rhabdovirus (22, 23). Soybean is an important crop in South Africa, where on average 190 000 tons are produced

annually, and investigation into diseases that may affect the yield of the crop is of considerable importance (www.sspcom.com/aboutsoy.htm).

The aims of this study were to identify, and partially characterize the putative rhabdovirus associated with the blotchy mosaic disease of soybeans in South Africa.

Materials and Methods

Virus sources and sample collection

Plants with the characteristic blotchy mosaic symptoms associated with the rhabdovirus infection and a range of similar mosaic-like symptoms were collected annually since 1993, during mid-summer from the major soybean production areas of Limpopo, Gauteng, Mpumalanga and North Western Province. Plants were immediately placed in labelled plastic bags and kept at 4°C until further tests were performed. Prior to the production of a virus-specific antiserum, leaf material was examined for viral particles as described by Hamilton et al (9) by negative staining with 2% uranyl acetate, pH 3.8 and examination with an ABT 002A electron microscope. Subsequently plants were tested by immuno-electron microscopy (IEM) as described below. For molecular analysis, symptomatic soybean leaves from a single site in the Brits/Thabazimbi area were stored at –70°C until RNA extraction or PCR was conducted.

Virus culture establishment and transmission

A number of methods for virus transmission were assessed in order to establish, maintain and propagate the virus within the laboratory.

Grafting: Side cleft, bottle and approach grafting (3, 7) using field-collected, infected soybean plants as the donor, were performed. Healthy soybean cultivars (cv.) Forrest or B66S10 were used as recipient plants. Grafted plants were maintained for six to eight weeks under insect-free conditions in a greenhouse under natural light conditions.

Insect transmission: Various leafhoppers, considered the most likely group of vectors due to their abundance, were collected from soybean fields in which the blotchy mosaic symptoms were prevalent. Live leafhoppers were sorted into morphologically identical groups (morphogroups), based on colour, shape and size, and each morphogroup separately exposed to healthy soybean cv. Forrest plants within insect proof cages. Plants were monitored for symptoms for a twelve week period. Following the appearance of symptoms after exposure to one leafhopper morphogroup, this insect was maintained in the laboratory on soybean Forrest or B66S10 plants in insect cages under a single 400W HPS SON light at a 14h/10h light/dark photoperiod at 22-28°C. All subsequent insect transmissions were performed with leafhopper individuals from this colony by exposing healthy insects to symptomatic plants for one week, before transferring them to healthy soybeans for three weeks and then removing the plants and treating them with an insecticide. *Peragallia caboverdensis* (18) was routinely identified in this colony (third author). Subsequently voucher material has been deposited in Biosystematics division of the ARC-Plant Protection Research Institute, Pretoria (South Africa) (Accession numbers CCDL21647 (4 specimens) CCDL21648 (3 specimens), CCDL21649 (12 specimens).

Mechanical transmission. Mechanical transmission was done as published previously (21). Four individuals of each species or cv. were inoculated with SbBMV. These were; *Arachis hypogaea* cv. Sellie (groundnut), *Beta vulgaris*, *Chenopodium*

amaranticolor, C. murale, C. quinoa, Datura stramonium, soybean cv. A5304, A5308, A5474, A7119, A7372, B66S10, Edgar, Forrest, Hutton, Highveld Top, Ibis, PNR577G, and PNR855*, Gomphrena globosa, Lupinus albus* cv. Kiev*, Lycopersicon esculentum* cv. Rhodade*, Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. langsdorfii, N. occidentalis, N. rustica, N. tabacum* cv.'s Samsun, A4, TL33 and OD86. *Phaseolus vul*garis cv.'s Black Turtle Soup, Bonus, Bountiful, The Prince, Top Crop and Redlands Greenleaf, *Pisum sativum*, *Tetragonia expansa, Triticum aestivum, Vicia faba, Vigna unguiculata* cv. Blackeye and *Zea mays*. Inoculated plants were kept in insect-free greenhouses and were monitored for symptoms over a six to eight week period and tested for virus by IEM.

Virus isolation and propagation.

Isolation of the rhabdovirus was by serial limiting dilution mechanical inoculation transfers. Virus was propagated on soybeans using leafhopper transmission or between *N. benthamiana* plants by mechanical transmission.

Virus purification and antiserum production.

Rhabdovirus infection, and a lack of other viruses, was confirmed by electron microscopy in individual field-collected soybean cv. Prima plants displaying blotchy mosaic symptoms before they were pooled and purified by the method of Adam et al., (1). Antiserum to the partially purified virus preparation was prepared by immunizing a New Zealand white rabbit with four, weekly intramuscular injections. Immunoglobulins (Ig's) were purified from antiserum with the use of carboxy methyl (CM) Affigel blue columns, according to the manufacturer's instructions (BioRad, USA). The $F(ab')_2$ fragment of purified Ig's were prepared by pepsin digestion (4).

The optimal concentrations of seroreagents of F(ab')2 ELISA (2) were determined using a checkerboard titration design (31). The optimal sero-reagent combination was chosen as that which gave the highest differentiation between healthy and infected plant material extracts.

Transmission electron microscopy

To determine the morphology and virion size, leaf dips of infected plants, were negatively stained and examined as described above. *Tobacco mosaic virus* (TMV) was used as an external standard to calibrate magnifications. Individual particle dimensions (n = 55) were then measured on electron micrographs, relative to TMV. To observe the intercellular particle distribution, embedded ultra-thin sections (30) of infected plant material were observed under a Philips EM301 TEM.

Immuno-electron microscopy was conducted as described by Roberts (28) with virus trapped on colloidion coated grids with a 1:100 dilution of the antiserum produced above.

RNA extraction and degenerate primer design

Viral RNA was extracted from infected plant material using the SV Total RNA Isolation System (Promega, Wisconsin) according to the method described by the manufacturers. Primer design was described previously (17). In short, ClustalW in BioEdit version 7.0.0 (8) was used to perform a multiple alignment of the L gene of various nucleorhabdoviruses (*Sonchus yellow net virus* (SYNV, Genbank accession number L32609), *Rice yellow stunt virus* (RYSV, AB00125), *Maize mosaic virus* (MMV, AY618418) and *Taro vein chlorosis virus* (TaVCV, AY674964)) and cytorhabdoviruses (*Lettuce necrotic yellows virus* (LNYV, NC007642), *Northern*

cereal mosaic virus (NCMV, NC002251) and *Strawberry crinkle virus* (SCV, AY250986)) available on Genbank. Degenerate primers were designed to relatively conserved regions of the L genes. These degenerate primers were expected to be broadly reactive to plant rhabdoviruses.

RT-PCR

For RT-PCR, conserved areas of known plant rhabdovirus L genes were targeted and degenerate primers RhabF (5'-GGATMTGGGGBCATCC-3') and RhabR (5'-GTCCABCCYTTTTGYC-3') were used from previous studies (17). The expected amplicon from this primer set was approximately 900 bp depending on rhabdovirus species detected. RT-PCR was carried out on total RNA extracted from symptomatic soybean leaves. For cDNA synthesis, 8.5 µl RNA and 2 µl RhabF (10 pmol) were incubated at 65 °C for 10 min and cooled on ice for 2 min. Then 5x Expand Reverse Transcriptase Buffer, 100 mmol/L DTT, 10mM dNTP's, 40 U RNase Inhibitor and Expand Reverse Transcriptase (Roche, Switzerland) were added and incubated at 43°C for 1 h. *Lettuce necrotic yellows virus* RNA was kindly supplied by R. Dietzgen (University of Queensland, St. Lucia, Australia) and cDNA synthesis of LNYV was also performed as mentioned above and used as a cDNA and PCR control. PCR reactions of 50 µl reaction volumes were prepared. The reaction mixture consisted of 5 µl of 10x reaction buffer (Bioline, UK), 50 mM $MgCl_2$, 40 mM dNTP's, 10 pmol of each primer, and 2.5U Taq polymerase (Bioline, UK). Finally 5 µl of the cDNA template was added, and the volume made up to 50 ul with UHQ water. The cycle conditions, on a ABI GeneAmp PCR System 2700 (PE Applied Biosystems, Connecticut), were as follows: one cycle at 94 °C for 2 min, and then 35 cycles of 94 °C for 30 s, 37 °C for 30 s, 72 °C for 90 s, followed by one final cycle at 72 °C for 7

min. Amplicons were analysed on a TAE buffered 1% agarose gel with a O'GeneRuler 200bp DNA Ladder Plus (Fermentas, Vilnius Lithuania) and viewed under an UV transilluminator (Vilber Lourmat, France). A SCV partial cDNA clone of a pBluescript plasmid containing the L gene sequence of SCV was kindly supplied by M. M. Goodin (University of Kentucky, Lexington) and was used as a PCR positive control. PCR products of ~900bp were purified from the gels using Wizard SV gel and PCR clean-up system (Promega, Wisconsin).

Sequencing and sequence analysis

Sequencing reactions were performed using ABI Prism BigDye Primer Cycle Sequencing Kits (PE Applied Biosystems, Connecticut) according to the manufacturer's specifications. Sequencing was done with an automated fluorescent sequencer (ABI 377 DNA Sequencer, PE Applied Biosystems, Connecticut) at the University of Pretoria's commercial sequencing facility. Amplicons were directly sequenced in both directions using RhabF and RhabR primers. The sequences were analysed by using the basic local alignment research tool (BLAST) program available on the National Centre for Biotechnology Information website (http://ncbi.nlm.nih.gov/). The partial L gene sequence obtained from soybean plants with the unidentified rhabdovirus was aligned and compared to the polymerase genes of available cytorhabdoviruses NCMV (NC002251), SCV (AY250986), LNYV (NC007642) and *Lettuce yellows mosaic virus* (LYMoV, accession number EF687738), as well as to available nucleorhabdoviruses RYSV (AB001125), SYNV (L32603), MMV (AY618418), *Maize fine streak virus* (MFSV, AY618417), TaVCV (AY674964), and the unassigned Orchid fleck virus (OFV, NC009609), Iranian maize mosaic virus (IMMV, DQ186554) and Cynodon rhabdovirus (CRV EU650683) by

doing a multiple alignment with ClustalX in BioEdit version 7.0.0 (8). From this alignment SbBMV was compared to the same cognate region of other plant rhadoviruses for analysis. The pairwise distance (p-distance) of cognate regions of the unidentified soybean rhabdovirus compared to known plant rhabdoviruses were calculated and a neighbour-joining phylogenetic tree was constructed using the Mega Version 3.1 (16).

Results

Virus sources and distribution of disease

A total of 1303 samples displaying the blotchy mosaic-like symptoms were collected. The vast majority of these were collected in either the Brits/Thabzimbi area (North West Province) or Groblersdal-Loskopdam area (Mpumalanga) (626 and 436 samples respectively) as the disease appeared more prevalent in these areas. Symptomatic plants were less prevalent in the Mpumalanga Lowveld and Limpopo Province where 74 and 109 samples respectively were collected. No examples of the typical blotchy mosaic symptoms were obtained from the Mpumulanga or Gauteng Highveld regions where 58 samples with other types of mosaic symptoms were collected. Samples were tested for virus by either negative staining followed by EM analysis (954 samples) or by IEM (349 samples). Rhabdovirus-like particles, at extremely low numbers were observed in 346 samples (36%) amongst those subjected to negatively-stained leaf dips (Figure 2 a, and b), while 227 samples (65%) had rhabdovirus-like particles at relatively low numbers in IEM tests. Flexuous rod-shaped particles were often observed in negatively stained preparations, especially with samples where the symptoms were described as an “atypical type of blotchy mosaic”. ELISA was conducted on 45 such plants to detect cowpea aphid borne mosaic virus, peanut mottle

virus and soybean mosaic virus, all previously found in the same districts where the collection of blotchy mosaic symptomatic plants took place. All of these were infected with soybean mosaic virus (results not shown). The rhabdovirus containing samples were restricted to the warmer, low-lying soybean production areas in the northern parts of South Africa, and were more prevalent in Brits/Thabazimbi, Northwest Province and Groblersdal/Loskopdam, Mpumalanga. None of the samples collected from the Highveld regions of Gauteng or Mpumalanga contained rhabdovirus-like particles

Virus establishment and transmission

Grafting of symptomatic field collected plants from the Loskopdam and Brits/Thabazimbi areas onto healthy soybean indicator plants was attempted with 66 samples (51 and 15 plants collected either early or late in the season respectively). Of these, 20 samples were grafted immediately in the field while the remaining 46 samples were made with cuttings in the laboratory. Only 8 successful graft unions were obtained, all from plants collected relatively early in the growth season and grafted in the laboratory with bottle grafting. In only three instances was transmission successful with the recipient soybean plants developing blotchy mosaic symptoms after 3-4 weeks and containing virus particles when tested by IEM.

The various leafhopper individuals collected from soybean fields were sorted into two prevalent morphogroups while the remainder made up a further 5 groups. The two prevalent leafhopper groups was *P. caboverdensis* (Cicadellidae; Agallinae; Agalliini), and an unidentified species of *Empoasca* (Cicadellidae; Typhlocybinae;Empoascini). Identification of the latter species was not possible due

to the limited taxonomic work of this group in the Afrotropical Region. Transmission of the rhabdovirus was achieved by allowing individuals of *P. caboverdensis* to feed directly on un-rooted cuttings for seven days, and then transferring them to healthy plants. The soybean indicator plants showed typical blotchy mosaic symptoms after two months, and virus particles could be detected in the plants by IEM. The *P. caboverdensis* colony was reared and maintained on soybean plants for three years, without the appearance of any blotchy mottle-like symptoms on these plants suggesting that the colony was non-viruliferous. Propagation of the virus by transmission to soybean plants using individuals from the *P. caboverdensis* colony was successful for two serial passages on three occasions with 20 plants per transmission, where-after the virus could no longer be transmitted. Transmission rates varied between 10 and 30%.

Mechanical transmission

Mechanical transmission of the rhabdovirus was achieved, following a large number of initially unsuccessful attempts. Successful mechanical inoculation was achieved with young symptomatic leaf material, collected very early in the growth season and macerated in cold 0.0l M phosphate buffer, pH 7.1, and immediately inoculated onto soybean cv. Forrest. Virus was transmitted to Forrest from five out of thirteen field collected samples. Further serial transmission to more Forrest plants was extremely inefficient with less than 10% of plants becoming infected. Further serial mechanical transmission was done for four passages, where after virus was lost from all but one line. This source (accession number 95/0131) was used for subsequent studies on alternate host plants, virus purification, particle morphology and distribution in the

cell. The virus was considered isolated as it effectively passed through four serial limiting dilution infections in which less than 10% of plants became infected.

Among the host plants tested, mechanical transmission of the virus from soybean could only be demonstrated to soybean cv.'s Forrest, Edgar and Ibis and to *Nicotiana benthamiana*. Mechanical transmission from soybean to soybean was inefficient with transmission occurring at frequencies less than 30%. Mechanical transmission from soybean to *N. benthamiana* and between individual *N. benthamiana* plants resulted in symptomless infected plants, but at transmission rates of 30 to 40%.

During virus purification a considerable loss of particles occurred at each step as determined by EM therefore only two rounds of differential centrifugation with a 20% (w/v) sucrose pad were conducted but the resultant partially purified virus was not placed on a final sucrose gradient. Six bleeds were obtained from a rabbit immunized with four injections of a partially purified virus preparation, but the antiserum was found not to be suitable for use in ELISA due to a high non-specific response to healthy plant components even following cross absorption of antiserum with healthy plant material (results not shown). The antiserum could however be used in IEM with virus particle enrichment being increased five-fold relative to pre-immune serum (Figure 2 c).

Virus morphology

Symptomatic soybean cv. Forrest plants were used to determine virus particle morphology and the intercellular distribution of the particles using transmission electron microscopy on ultra-thin sections of embedded material or negatively stained

leaf dips. Bullet-shaped virions were found in very low numbers in the cytoplasmic areas (Figure 3). The average (n=55) length of the virions as determined by negative staining of leaf preparations was 360nm, with a minimum length of 180nm and a maximum of 420nm, with most particles found in a range of 338-371nm without a clear modal length. The width varied between 79nm and 93nm (Figure 2). This size distribution falls within size range of the *Rhabdoviridae* (14).

Amplification and sequencing

The amplicon obtained with the primer set was a ~900 bp segment from SCV, LNYV and SbBMV (Figure 4). The PCR product was purified from the gel and directly sequenced in both directions utilizing RhabF and RhabR. RhabF proved to be a poor primer for sequencing and only the sequence generated by RhabR could be used and a 522 bp nucleotide sequence was obtained. BLAST analysis confirmed that the PCR amplicon was of viral RNA (polymerase gene) origin and that it was most closely related to known *Cytorhabdovirus* polymerase genes. The sequence was submitted to GenBank with the accession number EU877231.

Sequencing analysis

Pairwise distances of SbBMV to other polymerase genes of different plant rhabdoviruses were calculated using a cognate region of 410 bp with MEGA 3.1 (16). The SbBMV had the highest similarity to NCMV with 60.7 % and then LNYV and SCV (59.3% and 58.5% respectively) followed by the other plant rhabdoviruses (Table 2).

A neighbour-joining phylogenetic tree (Figure 5) was constructed with the same cognate region using Mega 3.1 (16). The tree separated the selected plant rhabdoviruses into two distinct clades, correlating with the *Cyto-* and *Nucleorhabdovirus* genera. Within this analysis, the SbBMV was found to be most closely related to the cytorhabdoviruses, especially NCMV.

Discussion

We have identified a new cytorhabdovirus associated at levels of 37 or 65% (depending on whether negative staining of leaf dips or IEM was used) with a blotchy mosaic disease of soybeans *(Glycine max)* in South Africa and propose the name soybean blotchy mosaic virus (SbBMV), in view of its apt description of the disease with which it is associated. The relatively low level of association is considered primarily due to the relatively low number of virus particles found in leaves, the relative insensitivity of both techniques as used, and the fact that a number of samples were collected which were not considered typical blotchy mottle symptoms. A number of the samples, particularly amongst those lacking rhabdovirus-like particles, contained flexuous rod-shaped particles, which in the case of those tested by ELISA were found to be particles of soybean mosaic virus, but could also include cowpea aphid-borne mosaic virus or peanut mottle virus, previously found on soybeans in these areas (23) causing some of the atypical blotchy mosaic- type symptoms (21). Future studies, using a specific primer pair in PCR, need to be conducted to ascertain the exact nature of the disease and associated with this virus. The disease is found annually during the growing season throughout the warmer low-lying soybean-producing areas of South Africa with incidences that vary from 0-32% in the individual fields. The size of the SbBMV particles fall within the range of those of other members of the *Rhabdoviridae*. The sizes ranged from180 to 420nm, with most between 338 and 371nm, and a width of 79-93nm (negatively stained preparations of infected plants). In ultrathin sections of leaves the virion particles were found in low numbers in the cytoplasm of infected soybean cells.

Identification and rapid detection of SbBMV has long been hampered by difficulties in establishing the virus in the greenhouse due to difficulties in transmitting the virus. However, during the current study, the development of an IEM technique with a relatively crude antiserum to virus partially purified from field-collected plant material, followed by the development of a generic rhabdovirus polymerase RT-PCR, was found to be a valuable tool for the maintenance and identification of this virus. An alternate propagation host to soybean viz. *Nicotiana benthamiana* was identified using IEM, but was difficult to work with as it was asymptomatic and individual inoculated plants required IEM tests to be conducted to ascertain their status. The virus was found to be mechanically transmissible between soybean plants, between soybean and *Nicotiana benthamiana* and amongst *N. benthamiana* plants at low transmission rates.

Because of the large numbers of leafhoppers observed in a local soybean field, and the fact that a number of other rhabdoviruses are known to be transmitted by these insects, we determined whether these insects could serve as vectors for SbBMV. One of two prevalent morphogroups amongst field collected individuals, later identified as *P. caboverdensis*, could transmit the disease to healthy soybeans. A non-viruliferous colony of this species was used to confirm the transmission under laboratory conditions. Further leafhopper species present locally may also serve as vectors and further studies are required to determine this along with transmission characteristics of *P. caboverdensis*. This species has been collected on a very wide range of plants in Southern Africa, such as Graminae and many dicotyleonous families (M. Stiller, personal observation). Its life cycle needs examination to determine disease and vector control protocols.

Sequence information for SbBMV generated during this study indicated that it is related to cytorhabdoviruses, particularly to NCMV. Based on the morphological and nucleotide sequence data, we concluded that the virus belongs to the genus *Cytorhabdovirus,* family *Rhabdoviridae,* and in view of its low sequence similarity with known rhabdoviruses, we propose that it be considered a new species, and be named soybean blotchy mosaic virus (SbBMV).

Amongst the known plant rhabdoviruses, only SCV (12, 24), CRV (17) and now SbBMV have been reported from South Africa. In view of the extensive indigenous flora in South Africa it is likely that many more plant rhabdoviruses may occur here. The universal rhabdovirus protocol used in this study could also be used as a tool to detect known, as well as new, plant rhabdoviruses in field studies, broadening our understanding about the epidemiology of these viruses. Thus far the degenerate primers RhabF and RhabR were employed to amplify four different plant rhabdoviruses, including the two new putative plant rhabdoviruses, SbBMV and CRV (17).

Further studies are needed to develop primers and a SbBMV specific RT-PCR to; 1) confirm the association of this virus with the disease, 2) to prove that the virus is the etiological agent of the disease, 3) to study the possibility that other leafhopper species may vector the virus, 4) to determine the natural alternate host of the virus during winter when soybean fields lie fallow, and 5) to better characterize the virus with regards its transmission, genome, translation and replication strategies. Methods

to control the disease can only be efficiently applied once a number of these aspects are known.

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Tables:

Table 1: Available sequences of plant rhabdoviruses on Genbank. The abbreviation, accession number as well as the reference of each of these viruses are also listed.

Unassigned *Rhabdoviridae*	Acc. Number	Reference
Orchid fleck virus (OFV)	NC009609	15
Cynodon rhabdovirus (CRV)	EU650683	17
Iranian maize mosaic virus (IMMV)	DQ186554	19
Nucleorhabdovirus		
Sonchus yellow net virus (SYNV)	L32603	5
Rice yellow stunt virus (RYSV)	AB001125	13
Maize mosaic virus (MMV)	AY618418	26
Maize fine streak virus (MFSV)	AY618417	25
Taro vein chlorosis virus (TaVCV)	*AY674964*	27
Cytorhabdovirus		
Lettuce necrotic yellows virus (LNYV)	NC007642	6
Lettuce yellow mottle virus (LYMoV)	*EF687738*	10
Northern cereal mosaic virus (NCMV)	NC002251	29
Strawberry crinkle virus (SCV)	AY250986	12, 24

Table 2: Similarity matrix of partial nucleotide sequences of the L gene of plant rhabdoviruses. Relative sequence similarity to SbBMV is shown in bold. The similarity matirix was calculated using Mega 3.1 (16).

	LNYV	MMV	NCMV	RYSV	SCV	SYNV	OFV	TaVCV	MFSV	LYMoV	SbBMV
LNYV											
MMV	52.2										
NCMV	59	50									
RYSV	50.7	56.3	53.7								
SCV	68.8	51	56.3	51							
SYNV	46.6	55.1	49.8	53.7	47.3						
OFV	48.8	55.6	54.1	54.6	49.3	59					
TaVCV	53.2	72	53.9	57.1	56.1	53.7	53.9				
MFSV	51.7	58.3	53.7	52.7	54.1	59.3	58.5	58.5			
LYMoV	73.9	50.5	58	53.9	70	47.6	50.5	51.5	52.2		
SbBMV	**59.3**	**49.8**	**60.7**	**53.4**	**58.5**	**49.5**	**51.5**	**51.7**	**51.7**	**56.8**	
CRV	50.7	69.7	51.5	56.3	53.9	54.4	51.7	71	57.3	51.2	**48.8**

Legends for Figures:

Figure 1. Typical blotchy mosaic symptoms with which SbBMV is associated.

Figure 2. Electron micrograph of symptomatic soybean leaf material subjected to negative staining (a and b) and showing a typical rhabovirus-like particle of SbBMV, (a) and nucleocapsid of SbBMV (b) or (c) Immuno-electron microscopy using antisera produced to SbBMV.

Figure 3. Electron micrograph of ultra-thin sections of embedded SbBMV infected soybean (Accession 95/0131) showing typical rhabovirus-like particles (arrows) occurring in the cytoplasm (CY) of cells. CP = chloroplast, CW = cell wall, A = amyloplast, Nu = nucleoplasm, Va = vacuole.

Figure 4. RT-PCR amplification of L gene fragment of SbBMV using degenerate primers. Samples were analysed on a 1% agarose gel and stained with EtBr. The O'GeneRuler 200bp DNA Ladder Plus (lane M), SCV (lane 1), LNYV (lane 2), SbBMV (lane 3) and negative, water control (lane 4) are indicated.

Figure 5. Phylogenetic tree of partial nucleotide sequences of SbBMV and other plant rhabdoviruses (Table 1). The tree was constructed using MEGA 3.1 using the neighbour-joining method (16). The abbreviations, accession number as well as the references are listed in Table 1.

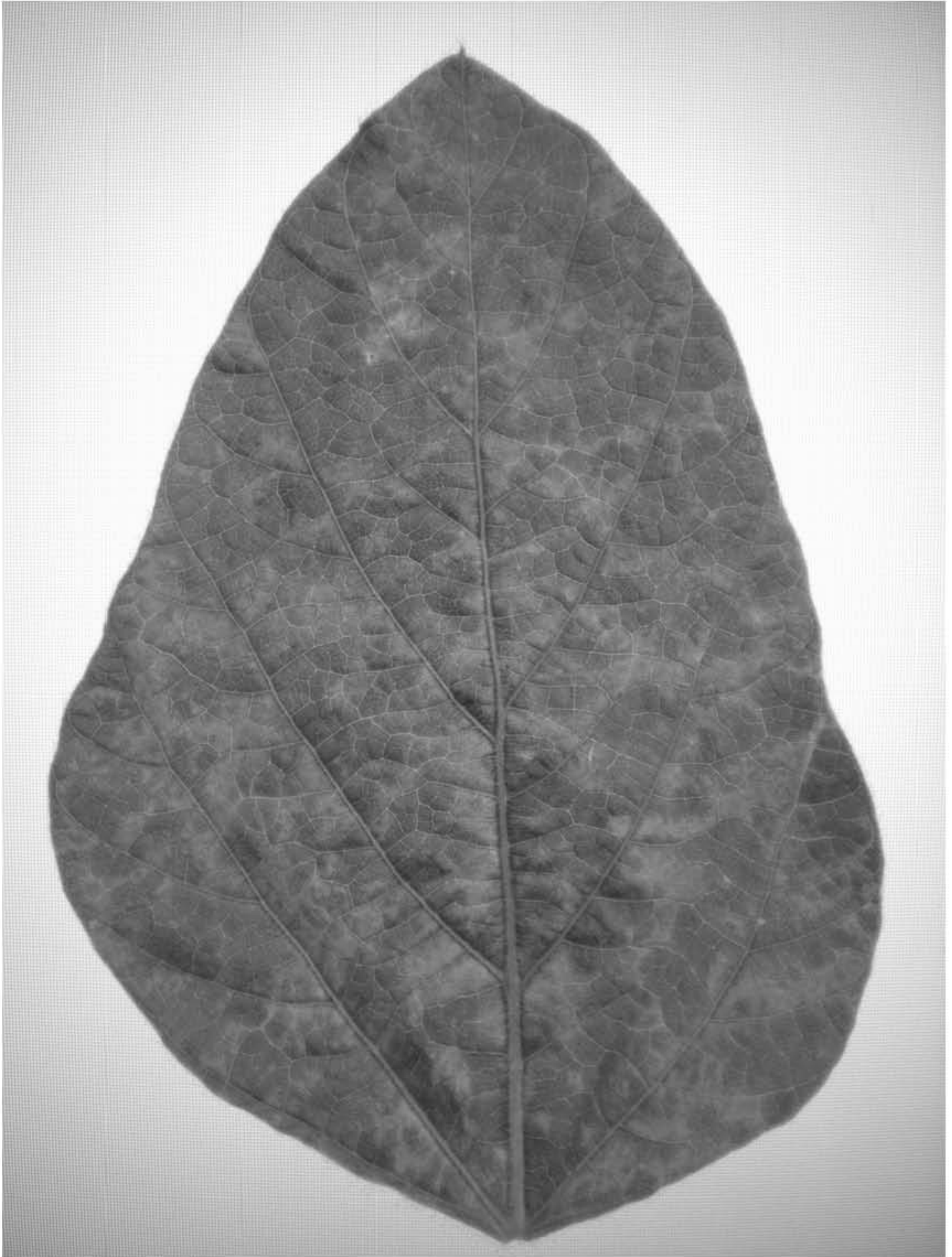

Fig. 1. Typical blotchy mosaic symptoms with which Soybean blotchy mosaic virus is associated.

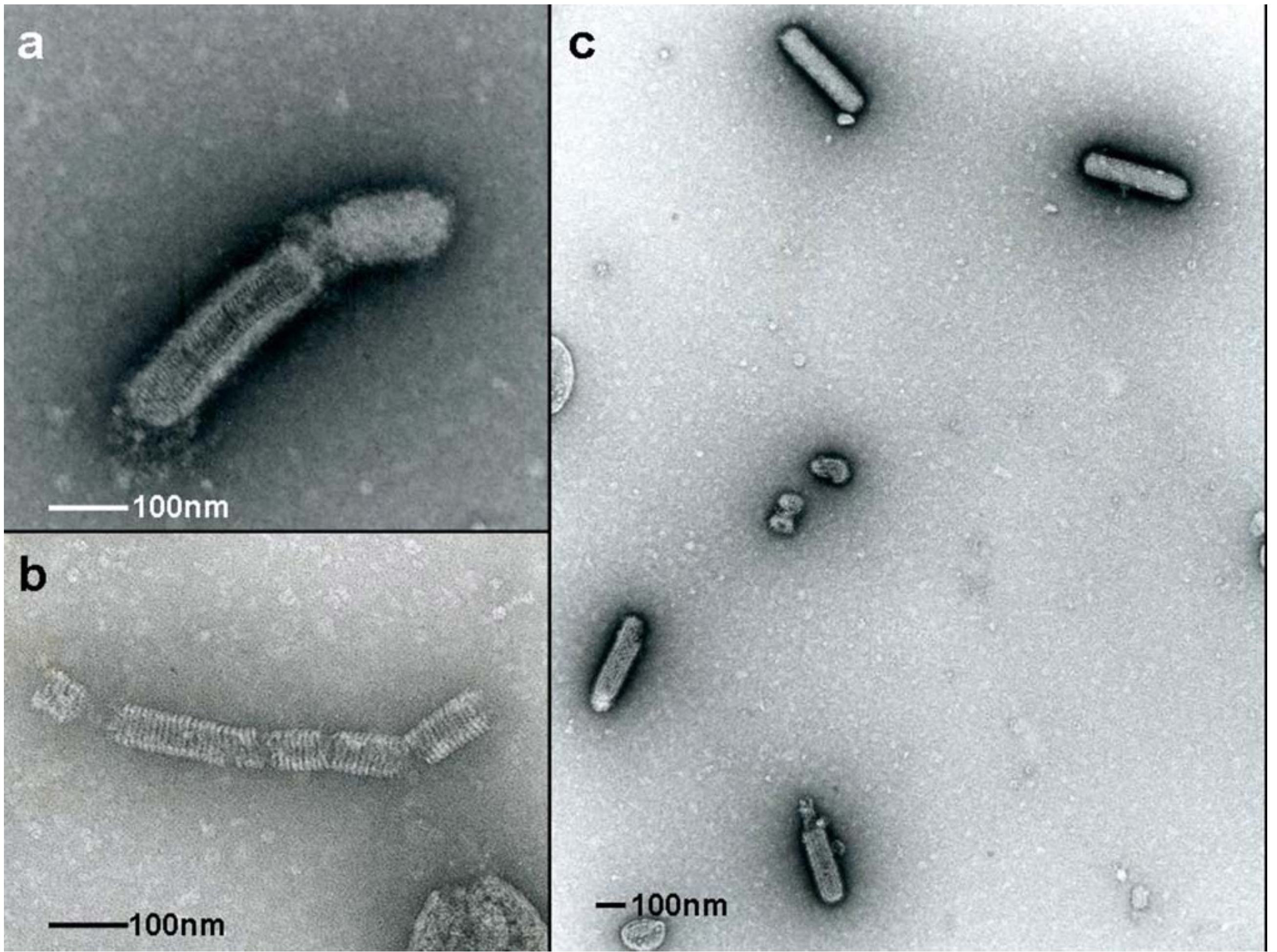

Fig. 2. Electron micrograph of **a** and **b,** symptomatic soybean leaf material subjected to negative staining and showing a typical *Rhabdovirus* sp.-like particle of **a,** Soybean blotchy mosaic virus (SbBMV) and **b,** nucleocapsid of SbBMV or **c,** immunoelectron microscopy using antisera produced to SbBMV.

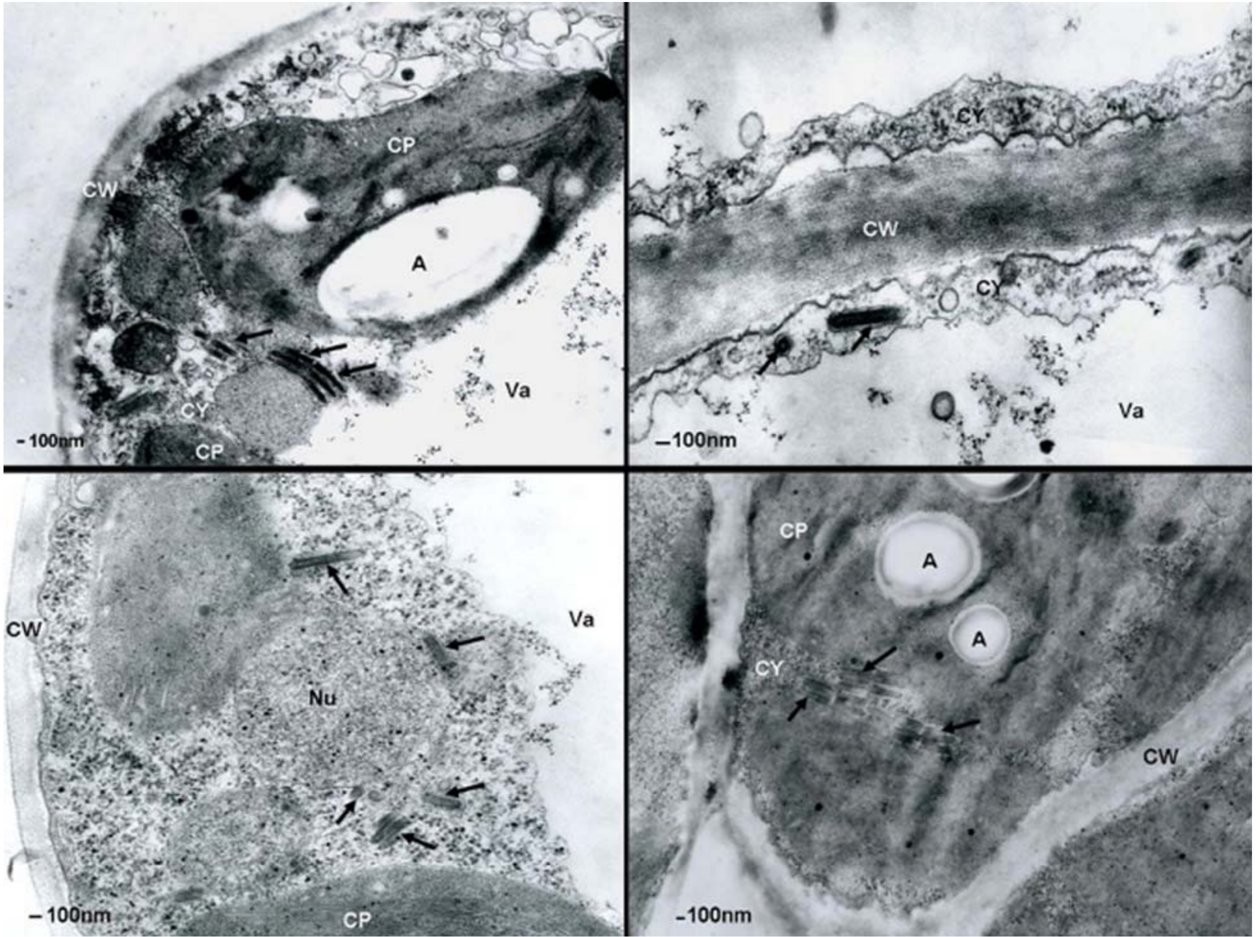

Fig. 3. Electron micrograph of ultrathin sections of embedded Soybean blotchy mosaic virus-infected soybean (accession no. 95/0131) showing typical *Rhabdovirus* sp.-like particles (arrows) occurring in the cytoplasm (CY) of cells. CP = chloroplast, CW = cell wall, A = amyloplast, Nu = nucleoplasm, Va = vacuole.

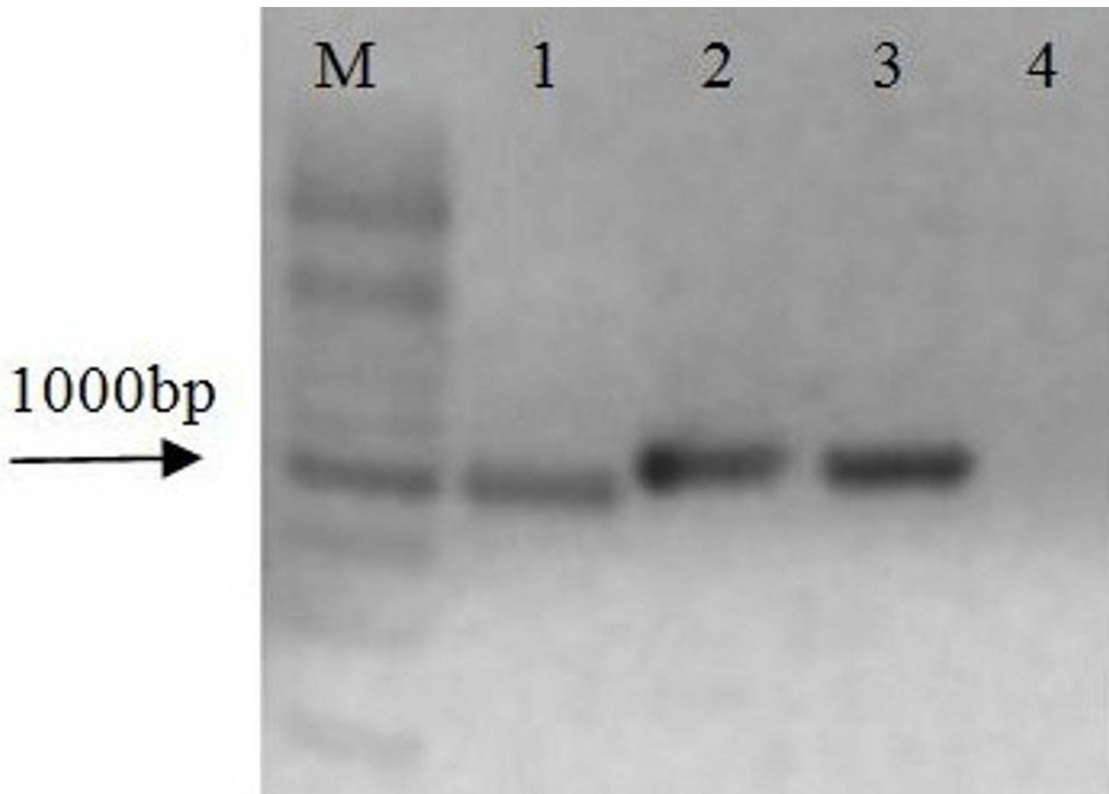

Fig. 4. Reverse-transcriptase polymerase chain reaction amplification of *L* gene fragment of Soybean blotchy mosaic virus (SbBMV) using degenerate primers. Samples were analyzed on a 1% agarose gel and stained with EtBr. Lane M, O'GeneRuler 200-bp DNA Ladder Plus; lane 1, *Strawberry crinkle virus*; lane 2, *Lettuce necrotic yellows virus*; lane 3, SbBMV; and lane 4, negative, water control.

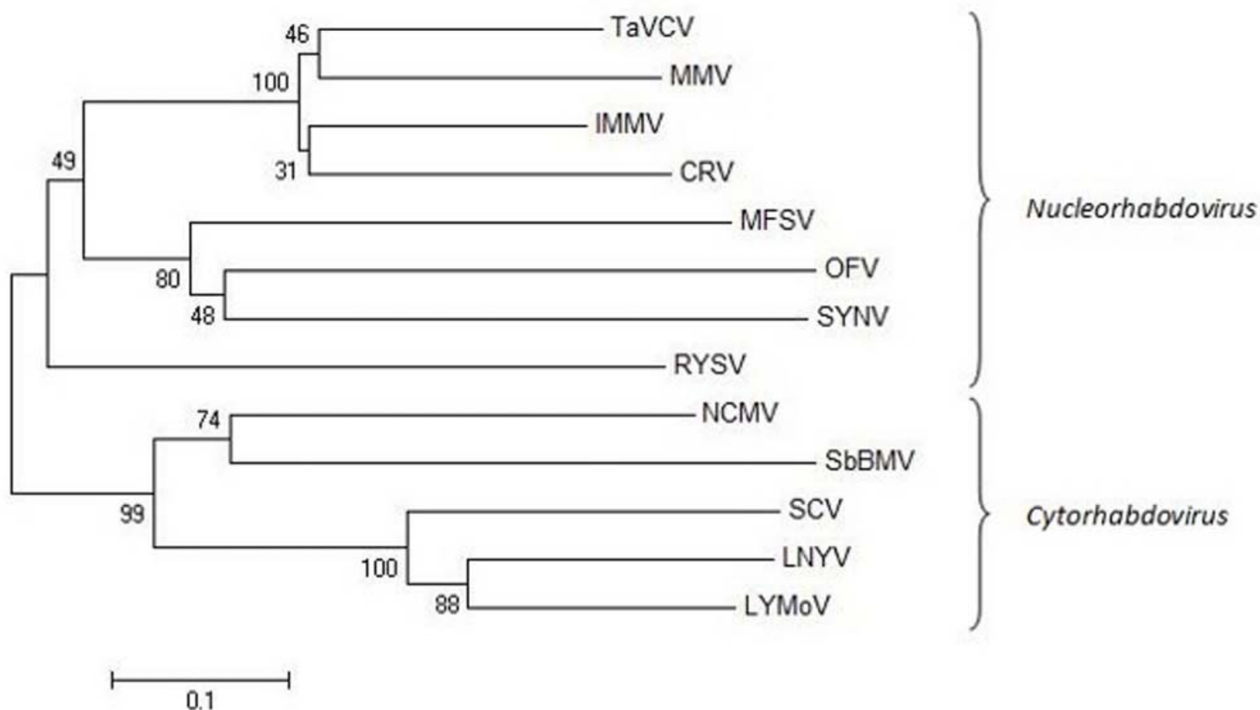

Fig. 5. Phylogenetic tree of partial nucleotide sequences of Soybean blotchy mosaic virus (SbBMV) and other plant *Rhabdovirus* spp. Tree was constructed using MEGA 3.1 using the neighbor-joining method (16). TaVCV = *Taro vein chlorosis virus*, MMV = *Maize mosaic virus*, IMMV = Iranian maize mosaic virus, CRV = Cynodon rhabdovirus, MFSV = *Maize fine streak virus*, OFV = Orchid fleck virus, SYNV = *Sonchus yellow net virus*, RYSV = *Rice yellow stunt virus*, NCMV = *Northern cereal mosaic virus*, SCV = *Strawberry crinkle virus*, LNYV = *Lettuce necrotic yellows virus*, and LYMoV =*Lettuce yellow mottle virus*.