Alternative hosts and seed transmissibility of soybean blotchy mosaic virus

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Abstract

Soybean blotchy mosaic virus (SbBMV) is an important virus of soybean in the warmer regions of South Africa. The presence of the virus is associated with blotchy mosaic symptoms on soybean leaves and significant annual yield losses. The virus is a member of the genus *Cytorhabdovirus* and persists between soybean growing seasons. In this study, multiple specimens of indigenous tree species, other crops and herbaceous weeds surrounding soybean fields with high disease incidences of SbBMV were tested for the presence of SbBMV by RT-PCR in order to determine whether the presence of alternative hosts facilitates the seasonal carry-over of the virus. Commercial soybean cultivars commonly grown in the region were also evaluated for seed transmissibility of the virus. A total of 487 accessions representing 27 different species were screened and one accession each of *Flaveria bidentis, Lamium amplexicaule* and *Gymnosporia buxifolia* tested positive for the presence of SbBMV and may serve as possible alternative hosts of SbBMV, allowing over-wintering of the virus when soybean is absent. Symptoms associated with SbBMV infection were not present in any of the 2, 829 seedlings collected from naturally infected SbBMV plants, and none of the 21 seedlings showing various abnormalities and tested by RT-PCR were positive. SbBMV does not appear to be seed transmissible in soybean at an incidence above that which numbers screened would have detected. It was concluded that the presence of alternative plant hosts, functioning as viral reservoirs during the soybean off-season might allow for the re-emergence of the disease early in the soybean production season each year. Future work will investigate the role of *Peragallia caboverdensis,* the leafhopper vector of SbBMV, and specifically the possible propagative transmission of the virus in the persistence of the disease.

Keywords: plant rhabdovirus, alternative hosts; seed transmissibility; soybean.

Soybean blotchy mosaic virus (SbBMV) is an economically significant viral pathogen of soybean in South Africa first identified in the lower lying soybean production areas in the northern and eastern parts of South Africa (Pietersen 1993; Pietersen et al. 1998). A high incidence of diseased soybean plants displaying the characteristic blotchy mosaic symptoms on leaves is commonly present early in the production season, with symptom severity declining with time. Surveys conducted in the early 1990s reported disease incidences ranging between 0% and 32%, leading to yield losses of up to 20% depending on the cultivar (Pietersen 1993). In a second study

conducted later in the 1990s, disease incidence had risen to between 10% and 50% in fields (Pietersen et al. 1998).

Typical of the cytorhabdoviruses, SbBMV has large, enveloped bacilliform particles, which are distributed in the cytoplasm of host cells (Lamprecht et al. 2010). RT-PCR amplification and sequencing of a 522 nt portion of the RNA-dependent RNA polymerase (L) gene confirmed that the new soybean virus was related to the cytorhabdoviruses, with the highest nucleotide similarity (60.7%) to northern cereal mosaic virus. The virus was successfully transmitted mechanically to both soybean and *Nicotiana benthamiana*, and the leafhopper *Peragallia caboverdensis* Lindberg (*Cicadellidae, Agalliinae*) identified as its insect vector (Lamprecht et al. 2010).

Rhabdovirus genomes are generally between 12.5 kb and 14 kb in size, with the monopartite, negative sense genomic RNA encoding five conserved structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and L protein (Dietzgen and Kuzmin 2012; Jackson et al. 2005). Rhabdoviruses in the genera *Nucleorhabdovirus* and *Cytorhabdovirus*, also encode one or more accessory genes, postulated to be involved in movement between cells and other unknown functions. Recently, the family *Rhabdoviridae* was expanded to include two additional plant-infecting genera, the *Dichorhavirus* and *Varicosavirus*, which have bipartite genomes (Amarasinghe et al. 2017).

Despite the annual nature of soybean production, this disease of soybean persists between soybean growing seasons, as symptoms are present early in each new growing season. Possible mechanisms allowing for the carry-over of the virus between seasons include the presence of alternative plant hosts in areas surrounding soybean fields, presence of soybean volunteer plants during winter and/or replicative transmission of the virus by its insect vector. Seed or pollen transmission is another method by which the virus can be introduced each year, especially as high incidences of the disease are often observed early in the growing season.

Virus transmission through seed has been observed in at least 25 groups of plant viruses, with approximately 18% of all plant viruses reportedly transmitted through seed (Johansen et al. 1994; Maule and Wang 1996; Mink 1993). Although only a small percentage of infections result in eventual seed transmission, virus propagation through this mechanism is significant as a result of the introduction of new viral sources for further spread through arthropod vectors. Seed

transmission, however, plays a significant role in the epidemiology of only a few plant viruses such as bean common mosaic virus in beans (Morales and Castano 1987), soybean mosaic virus in soybean (Bowers and Goodman 1979), lettuce mosaic virus in lettuce (Dinant and Lot 1992), cucumber mosaic virus (CMV) (Johansen et al. 1994; Mink 1993), and the maize lethal necrosis complex (Mahuku et al. 2015; Zhang et al. 2011), but to date seed or pollen transmission of rhabdoviruses has not been reported (Jackson et al. 2005).

In this study, the presence of alternative plant hosts and seed transmissibillity of SbBMV was investigated for the first time. Asymptomatic indigenous trees, other crop plants and herbaceous weeds commonly present in areas surrounding fields where high disease incidences are observed each year were sampled and tested for the presence of SbBMV by RT-PCR. Leaf samples were collected from trees, crops, grasses and other herbaceous weeds in close proximity to commercial soybean fields with a disease incidence of at least 30% in the Brits area of the North West province in South Africa. Leaf material was collected at multiple sites on a plant, and each sample was assigned a unique accession number and GPS coordinates were documented for trees. Voucher specimens were collected for all plant species surveyed, and were identified by expert botanists in the Department of Plant Sciences, University of Pretoria, South Africa. Samples were collected in both the soybean production and off-season, and leaf material was stored in plastic bags at 4°C prior to being processed.

Pooled leaf samples (pieces from multiple individual leaves) were homogenized in liquid nitrogen, and total RNA extracted according to the method described by White et al. (2008) with a few modifications which included the omission of spermidine from the extraction buffer, and the use of 1.8 ml heated extraction buffer per sample. All centrifugation steps on the first day were performed for 15 min, and RNA pellets were washed with 500 µl 70% ethanol. The plant gene ribulose 1,5-biphosphate carboxylase (RuBisCo) was amplified from RNA of all accessions using the rbcLa primer pair to ensure RNA quality is sufficient for RT-PCR analysis as polysaccharides, polyphenolics and other secondary metabolites associated with woody tissues can bind or co-precipitate RNA, lowering RNA quality and leading to false negative results (White et al. 2008). cDNA synthesis was done using the M-MLV Reverse Transcriptase system (Promega, Madison, USA) according to the manufacturer's instructions with two modifications: 5 µL random hexamer primer (4 µM primer in final reaction) was allowed to anneal to 2 µl total RNA. This was followed

by the addition of 5.5 µl master mix consisting of 2.5 µl M-MLV RT 5x Reaction buffer (Promega), 1.25 µl dNTPs (10 mM each dATP, dCTP, dGTP, dTTP) (Kapa Biosystems, Wilmington, USA), 0.125 µl (25 U) M-MLV RT (Promega) and 1.6 µl molecular grade water added for cDNA synthesis. Each 12.5 µl PCR amplification reaction contained 2.5 µl 5x MyTaq Reaction buffer (Bioline, London, UK), 0.25 µl (0.2 µM) each of rbcLa F (5' ATGTCACCACAAACAGAGACTAAAGC 3') (Levin et al. 2007) and rbcLa R (5' GTAAAATCAAGTCCACCRCC 3') (Kress and Erickson 2007), 0.125 µl (0.6 U) MyTaq DNA Polymerase (Bioline), 1.5 µl cDNA and molecular grade water to 12.5 µl. The PCR program consisted of 95°C for 1 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 15 sec, 72°C for 10 sec, and a final extension step at 72°C for 10 min. PCR products were separated by agarose gel electrophoresis on ethidium bromide-stained gels (1%), and visualized using UV light.

SbBMV was detected in leaf samples by RT-PCR using the Soyblotch F (5' CTTTGCCCAACTGGACTCCC 3') and Soyblotch R (5' TCCAAACAGTCTTCCCAGGC 3') primer pair designed to amplify a 354 bp portion of the SbBMV L gene, for which sequence information was available on the National Centre of Biotechnology Information (NCBI) database (EU877231). RT-PCR reactions were done in duplicate. cDNA synthesis was performed using the M-MLV Reverse Transcriptase system (Promega) as described above, with the Soyblotch F primer at 4 µM final concentration. PCR amplification was done using the MyTaq system (Bioline) as described above using the Soyblotch F and Soyblotch R primer pair, and an annealing temperature of 58°C. Soyblotch PCR products were purified for direct sequencing through the addition of 5U Exonuclease (Thermo Scientific, USA) and 1U FastAP Alkaline Phosphatase (Thermo Fisher, Waltham, USA) to 7 µl PCR product, and incubated at 37°C and 85°C for 15 min each. Each 10 µl sequencing reaction consisted of 3 µl template, 1 µl BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, USA), 2.25 µl of 5x Sequencing buffer (Applied Biosystems) and 2 µM (0.75 µl) of the Soyblotch F and Soyblotch R primers respectively. Sequences were subjected to a Basic Local Allignment Search Tool (BLASTn) search in NCBI in order to confirm presence of SbBMV.

Table 1. List of plant species collected and results of PCR tests for soybean blotchy mosaic virus.

Botanical name	Common name	Nr positive/nr tested
Amaranthus hibridus (A)	Smooth pigweed	0/29
Bidens pilosa (A)	Blackjack	0/16
Celtis africana		0/17
Chenopodium album	Goosefoot/lambsquarters	0/16
Chenopodium corinatum (A)	Creeping goosefoot	0/33
Cyperus spp.	-	0/23
Datura stramonium	Jimson weed; Thorn apple	0/31
Dichrostachys cinerea (P)	Sickle bush	0/15
Dinebra retroflexa (A)	Viper grass	0/25
Euphorbia heterophylla	Poinsettia	0/20
Flaveria bidentis (A)	Smelter's bush; Yellowtops	1/27
Gymnosporia buxifolia (P)	Spike thorn	1/26
Ipomoea purpurea	Morning glory	0/5
Lamium amplexicaule	Henbit	1/10
Malvastrum coromondelianum	False mallow	0/13
Medicago sativa	Lucerne	0/29
Portulaca quadrifida (A)	Chickenweed	0/32
Rapistrum rugosum	Bastard cabbage	0/24
Searsia leptodictya (P)	Mountain karee	0/10
Senecio abiifolius	-	0/3
Senegalia caffra (P)	Hook thorn	0/10
Sonchus oleraceus (A)	Sowthistle	0/9

Sonchus spp	Sowthistle	0/2
Symbrium thellungi	-	0/3
Tithonia rotundifolia (A)	Red sunflower	0/12
Vachellia karroo (P)	Sweet thorn	0/37
Vachellia tortilis (P)	Umbrella thorn	0/10

[P]Perrenial, [A]Annual

A total of 487 specimens representing 27 different species were collected in the soybean growing season in and surrounding fields where the disease incidence exceeded 30% (Table 1). Of these, two accessions, one each of *Flaveria bidentis* and *Gymnosporia buxifolia* consistently tested positive for the presence of the virus. Some samples were also collected during the South African winter months, from the same sites where samples had been collected during the soybean growing season. In this period, maize or wheat is commonly grown in rotation with soybean in the Brits area in South Africa. The accession of *L. amplexicaule* which tested positive for the presence of SbBMV was collected during winter, confirming the presence of the virus in an alternate host in the absence of soybean.

Two of the three new putative hosts identified for SbBMV were species of common weed. Weeds can act as pests, vector pathogens or act as reservoir hosts for viruses and insects as in the case of *L. amplexicaule* and *F. bidentis* and SbBMV (Wisler and Norris 2005). The higher genetic diversity of weeds in comparison to crops, the presence of numerous different weed species in an area, which limits contact between identical, susceptible plants, and long term selection of tolerance or resistance often results in asymptomatic virus infection in weed hosts. As a result of the establishment of a new monoculture crop, pathogens which were present asymptomatically at low levels in weeds may jump host to the new introduced crop, resulting in a symptomatic infection (Wisler and Norris 2005). It is possible that SbBMV is re-introduced onto soybean annually in this manner.

L. amplexicaule is a winter annual which germinates in late summer and flowers and dies the following spring or summer, and may thus act as a "bridge" for SbBMV between successive soybean seasons. *L. amplexicaule* has also previously been reported to serve as a naturally infected

weed host for plant viruses such as turnip yellows virus (Stevens et al. 2008), CMV (Nitzany 1975), and turnip mosaic virus (Stobbs and Stirling 1990). Potato virus Y and CMV have also been detected in other species in the genus such as *Lamium purpureum*, indicating that members of the genus may commonly be hosts to plant viruses (Kaliciak and Syller 2009; Tomlinson et al. 1970). *F. bidentis* is a member of the *Asteraceae* family, and considered an annual weed, but may survive the winter months in warmer areas such as Brits, where samples were collected. In contrast to *L. amplexicaule*, *F. bidentis* does not appear to be a common alternative host for plant viruses, but reports of CMV on *F. bidentis* are present in literature (Swanepoel and Nel 1995).

Gymnosporia buxifolia is an indigenous tree species commonly found in those areas where high incidences of the blotchy mosaic symptom is found, such as the Brits, Loskop Irrigation Scheme and Groblersdal areas in South Africa. As with *L. amplexicaule*, *G. buxifolia* is a member of the family *Fabaceae* (*Leguminosae*), and it was considered likely that SbBMV could move from one leguminous host to the next. The perennial nature of *G. buxifolia*, in contrast to the annual or short-lived perennial weeds *L. amplexicaule* and *F. bidentis* might render it a more effective and permanent reservoir host.

Seed from naturally infected, RT-PCR positive commercially grown soybean plants were also collected and planted to assess the seed transmissibility of the virus. This disease of soybean emerges early in the summer soybean growing season each year following the soybean off-season in the winter months. The early appearance of symptoms in a season is commonly observed when viruses are seed transmissible. 11, 6, 10 and 14 soybean plants of the soybean cultivars PHB 94Y80R (Pioneer), PHB 94Y20R (Pioneer), NS 7211R (Klein Karoo Seed Marketing) and 6.15F (Southern Hemisphere Seeds) respectively displaying the characteristic blotchy mosaic associated with SbBMV infection in fields were marked, and leaf material collected. The presence of SbBMV in leaf material was confirmed by RNA extraction and RT-PCR using the Soyblotch primer pair as described above. Seed was harvested once the pods had dried off, inoculated with *Rhizobium* spp. and grown in steam-sterilized potting mix under insect-free greenhouse conditions. Seedlings were watered approximately three times per week, and were visually evaluated for symptoms when they had reached at least the trifoliate stage. Seedlings displaying any virus-like symptoms or abnormalities were tested for SbBMV by RT-PCR using the Soyblotch RT-PCR system.

A total of 2, 829 seedlings representing four commercial cultivars commonly grown in the warmer, low-lying soybean production areas in South Africa were evaluated for symptoms. For each of the soybean cultivars 6.15F, PHB 94Y20R, PHB 94Y80R and NS7211R, 983, 125, 965, and 756 seedlings were screened respectively. The characteristic blotchy mosaic typically associated with the presence of SbBMV (Figure 1A) was not observed in any of the seedlings (Figure 1B). None of the 21 seedlings displaying various abnormalities and tested by RT-PCR were positive for SbBMV. SbBMV does not appear to be seed transmissible in these soybean cultivars at levels high enough to be detected given the numbers tested. This was expected, as no rhabdoviruses have been shown to be seed transmissible to date (Jackson et al. 2005; Dietzgen et al. 2017).

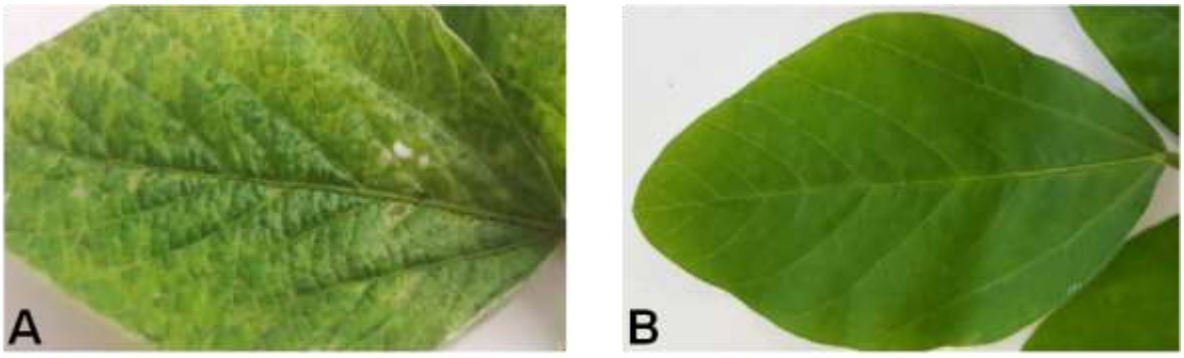

Figure 1. Photographs illustrating A) the typical blotchy mosaic associated with soybean blotchy mosaic virus on soybean leaves, and B) absence of symptoms on accessions grown from seed collected from diseased soybean mother plants in the field.

In future, control strategies against SbBMV should focus on the management of sources of inoculum from which the virus can be spread to soybean by *P. caboverdensis*. Weeds and volunteer plants can be eliminated, and crop residues destroyed (Wisler and Norris 2005). Removal of Johnson's grass, an alternative host of Maize dwarf mosaic virus (MDMV) through the application of herbicides increased yields of corn (Mark and Harold 1993). Treatment of perennial hosts such as trees with systemic insecticides will prevent *P. caboverdensis* and any other possible insect vectors from re-infesting soybean. The apparent lack of seed transmissibility of SbBMV implicates *P. caboverdensis* as the main route by which the virus spreads. Future work should include further characterization of the role of *P. caboverdensis* in the epidemiology of SbBMV, such as

associations with alternative hosts and mode of transmission of SbBMV, as well as the presence of additional insect vectors.

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