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**EFEITOS DO CARLAVÍRUS *Cowpea mild mottle virus* EM CULTIVARES
DE SOJA**

Botucatu

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DE SOJA**

Dissertação apresentada à Faculdade de Ciências Agronômicas da Unesp Câmpus de Botucatu, para obtenção do título de Mestre em Agronomia (Proteção de Plantas).

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TÍTULO DA DISSERTAÇÃO: EFEITOS DO CARLAVÍRUS Cowpea mild mottle virus EM CULTIVARES DE SOJA

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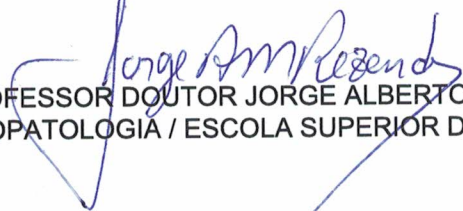
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Aos meus amados pais,

Lidia e Adhemar,

Dedico

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Autor Desconhecido

RESUMO

Doenças causadas por vírus são importantes fatores contrários a produção de soja. Entre elas, a doença da necrose da haste causada pelo *Cowpea mild mottle virus* (CPMMV) e transmitida pela mosca-branca *Bemisia tabaci* já foi observada em todas as principais regiões produtoras de soja do Brasil e a queima do broto (*Tobacco streak virus* – TSV) que têm ocorrência mais restrita nas regiões dos Estados do Paraná e de São Paulo. Os impactos causados por ambas doenças à sojicultura brasileira ainda são desconhecidos. Nesse contexto, o objetivo do presente trabalho foi avaliar os efeitos do CPMMV nas cultivares de soja mais utilizadas nas principais áreas produtoras da região sudeste e centro-oeste do Brasil e avaliar, por sequenciamento de nova geração (NGS) a ocorrência de vírus em uma área comercial de soja na região de Mogi Mirim. Para avaliar os efeitos do CPMMV em cultivares de soja, os experimentos foram conduzidos durante a safra 2017/2018 em quatro regiões: Botucatu e Mogi Mirim no estado de São Paulo, Pedra Preta no estado do Mato Grosso e Planaltina no Distrito Federal. O delineamento experimental foi feito em blocos casualizados, com dois tratamentos: plantas infectadas artificialmente com CPMMV e plantas saudáveis, e cinco repetições. As parcelas compreenderam 6 linhas (5 m), 0,45 m entre linhas e uma média de 14 plantas por metro. Seis cultivares comerciais foram utilizadas e distribuídas de acordo com a frequência que elas são utilizadas nessas regiões. Para a infecção artificial, o vírus foi transmitido via tampão fosfatado 0.01 M, pH 7 em 200 plantas por parcela. A presença do vírus nos dois tratamentos foi avaliada através de técnicas de análise molecular usando primers específicos para o CPMMV. Foram avaliados: altura da planta na época da colheita, número de vagens por plantas, peso de mil grãos (g) e produtividade. Embora algumas variedades testadas apresentaram-se assintomáticas à infecção do vírus, o CPMMV reduziu significativamente os parâmetros agrônômicos avaliados, causando perdas na produtividade de 174 a 638 kg ha⁻¹ sacos por hectare, dependendo da cultivar. Evidência de transmissão do vírus por sementes também foi observada. Para a detecção e identificação de vírus presentes em uma área comercial de soja na região de Mogi Mirim, o estudo com sequenciamento de última geração identificou infecção mista de ilarvirus/carlavirus e carlavirus/tospovirus. Os resultados desse trabalho sugerem que o CPMMV pode ter um importante papel na redução da produtividade

da soja no Brasil e infecções assintomáticas podem estar mascarando o atual impacto desse patógeno na cultura. O uso de sequenciamento de última geração como ferramenta nos estudos de doenças que afetam a cultura da soja se mostrou eficaz para identificar vírus insuspeitos.

Palavras-chave: Doenças de soja. Necrose da haste. Queima do broto. CPMMV. TSV. Transmissão.

ABSTRACT

Diseases caused by virus are important constraints to soybean production. Among them, stem necrosis caused by *Cowpea mild mottle virus* (CPMMV) and whitefly-*Bemisia tabaci* transmitted were observed in all the main soybean producer areas in Brazil and the bud blight (*Tobacco streak virus* – TSV) occurs strictly in regions Parana and Sao Paulo States. The impacts caused by both diseases on soybean production are still unknown. Therefore, the goal of this study was to evaluate the effects of CPMMV on the major soybean cultivars used in the main growing areas of the Southern and the Midwestern regions of Brazil, and evaluate through next generation sequencing (NGS) the presence of virus in a commercial field of soybean in Mogi Mirim County. To evaluate the effects of CPMMV in soybean cultivars, the experiments were conducted during the 2017/2018 season in four regions: Botucatu and Mogi Mirim Counties, in the state of Sao Paulo, Pedra Petra County in the state of Mato Grosso and in Planaltina County, in the Federal District. The experimental design was completely randomised, with two treatments (plants infected by sap transmission and healthy plants) with five replications. The plot was comprised of six rows (5m), 0.45 m between rows and an average of 14 plants per meter. Six commercial cultivars were used and distributed according to the frequency that they are planted in those regions. For the field inoculation, the virus was readily sap-transmitted in soybean plants 30 d after sowing using infected leaves ground in phosphate buffer 0.01 M, pH 7, using approximately two hundred soybean plants per plot. The presence of the virus was detected by RT-PCR using primers specific for CPMMV. Plant height (cm), number of pods per plant, 1.000-grain weight and grain yield were evaluated during harvest time. Although some cultivars tested presented asymptomatic infection for the virus, CPMMV reduced significantly the agronomic traits evaluated, causing losses in the yield ranging from 3.9 to 10.7 bags per hectare, depending on the cultivar. Evidence of seed transmission of CPMMV was also observed. For the detection and identification of viruses presence in a commercial field in Mogi Mirim County, the study with next generation sequencing identified mixed infection of ilarvirus/carlavirus and calarvirus/tospovirus. The results of this study suggest that CPMMV can have an important role in yield reduction of soybean in Brazil, furthermore, asymptomatic infections might be hiding the actual impact of this pathogen in this crop. The use of

next generation sequencing as a tool in the studies of viral diseases that affects the soybean has showed to be a powerful to detect unsuspected viruses.

Keywords: Soybean disease. Stem necrosis. Bud blight. CPMMV. TSV. Transmission

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INTRODUÇÃO GERAL

A soja [*Glycine max* (L.) Merrill] é uma cultura de grande importância econômica no Brasil, com um mercado interno de R\$ 2.7 bilhões e de US\$ 31 bilhões de exportação. O país é o segundo maior produtor mundial do grão, produzindo 114,095 milhões de toneladas em uma área cultivada de 33,914 milhões de hectares na safra 2016/2017 (HIRAKURI & LAZZAROTTO, 2014; CONAB, 2018). Na última década, houve um acréscimo em torno de 63,9% da área cultivada do grão no Brasil, com aumento da produtividade de 2.823 kg ha⁻¹ para 3.363 kg ha⁻¹ (CONAB, 2018).

Diversas doenças causadas por fungos, bactérias, nematoides e vírus podem comprometer a produção de soja. Já foram identificadas cerca de 40 doenças na cultura da soja no Brasil (EMBRAPA SOJA, 2013). No mundo, ao menos 34 viroses foram descritas infectando naturalmente a soja, e no Brasil, há relatos de pelo menos 15 delas (ALMEIDA, 2008; DE MARCHI *et al.*, 2018). Os vírus descritos em soja no Brasil pertencem aos gêneros: *Alfamovirus*, *Begomovirus*, *Carlavirus*, *Comovirus*, *Ilarvirus* e mais recentemente também um orthotospovirus foi descrito nesta cultura (DE MARCHI *et al.*, 2018; FONTES *et al.*, 2019).

Há poucos estudos no Brasil acerca dos principais prejuízos ocasionados por estes vírus, e os trabalhos publicados são, em geral, do final dos anos 1990 e início dos anos 2000. O melhoramento de soja é bastante dinâmico e novos cultivares são lançados anualmente visando aumento do potencial produtivo e resistência a doenças. Deste modo, este trabalho teve como finalidade estudar o impacto da espécie *Cowpea mild mottle virus* – CPMMV (*Carlavirus*, *Betaflexiviridae*) na produção dos cultivares atualmente mais plantados de soja nas regiões Sudeste e Centro-Oeste do Brasil (Capítulo 1), bem como identificar os vírus presentes em uma área comercial de soja na região de Mogi Mirim, com a detecção por sequenciamento de última geração. Foi constatada a infecção mista de ilarvirus/carlavirus e carlavirus/tospovirus (Capítulo 2). No capítulo 3 estão descritos os principais sintomas atualmente associados ao CPMMV em soja, uma vez que o CPMMV, cuja doença é conhecida como necrose da haste, não ocasiona mais este sintoma típico nos cultivares atualmente plantados no Brasil e sua presença no campo pode estar sendo subestimada.

REVISÃO DE LITERATURA

A cultura da Soja e suas doenças virais

Considerada uma das culturas mais antigas, a soja cultivada (*Glycine max* [L.] Merrill) originária do leste da Ásia, chegou ao ocidente no final do século XV e início do século XVI. Com o aumento de sua importância e do comércio, essa leguminosa foi levada para o sul da China, Coréia, Japão e sudeste da Ásia. No Brasil, o primeiro relato da introdução da soja foi feito no Estado da Bahia (DUTRA, 1882), mas foi nos estados do sul que essa cultura se expandiu de forma comercial (MIYASAKA; MEDINA, 1981).

A expansão da cultura da soja no Brasil ocorreu por vários fatores, tais como obtenção de genótipos adaptados a regiões de baixa latitude, e resistência a doença (SPECHT *et al.*, 2014). Assim, observou-se um incremento de produção partir do ano de 1970, passando de 1,5 milhão de toneladas para 119,281 milhões de toneladas em 2018, em uma área cultivada de 35 milhões de hectares (CONAB, 2018).

A soja é afetada por diversas doenças, das quais ao menos 14 doenças associadas a vírus foram descritos no Brasil infectando-a naturalmente (ALMEIDA, 2008). Segundo este mesmo autor, as doenças de maior impacto para a cultura da soja são: o mosaico comum da soja (espécie *Soybean mosaic virus* (SMV), gênero *Potyvirus*), a necrose da haste (espécie *Cowpea mild mottle virus* (CPMMV), gênero *Carlavirus*) e a queima do broto (espécie *Tobacco streak virus* (TSV), gênero *Ilarvirus*). Recentemente foi detectada também a espécie *Groundnut ringspot orthotospovirus* (GRSV) pertencente ao gênero *Orthotospovirus* infectando naturalmente soja e pouco ainda se sabe sobre seu impacto na cultura (DE MARCHI *et al.*, 2018; FONTES *et al.*, 2019).

O SMV, tem distribuição global, além de ser facilmente transmitido por sementes provenientes de plantas infectadas e por várias espécies de afídeos (ALMEIDA & MIRANDA, 1979; LIMA NETO & COSTA, 1979). Os principais danos ocasionados pelo SMV são a redução no rendimento e manchas no tegumento da semente, causando seu sintoma típico, conhecido por mancha café. Em 1994, 64% das cultivares de soja utilizadas por produtores brasileiros eram suscetíveis ao SMV (ALMEIDA *et al.*, 1994). Apesar dos relatos da variabilidade de estirpes do SMV e de quebra de resistência (SILVA *et al.*, 2003), não há informações de perdas associadas

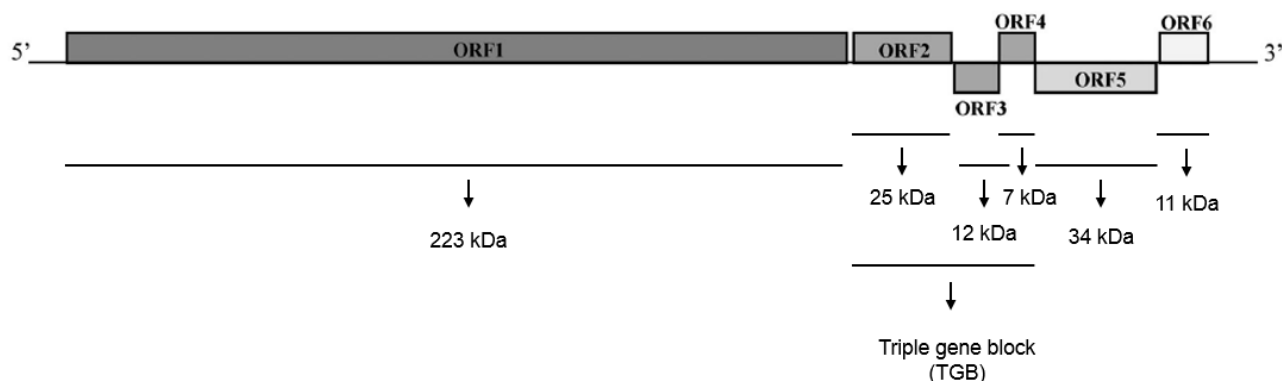
a este vírus atualmente no Brasil, uma vez que as cultivares comerciais apresentam resistência a ele.

A seguir serão abordados os gêneros *Carlavirus* e *Ilarvirus* foco de estudo da presente dissertação.

Gênero *Carlavirus*, espécie *Cowpea mild mottle virus*

O gênero *Carlavirus* compreende 53 espécies sendo o mais numeroso em termos de espécies na família *Betaflexiviridae* (ICTV, 2019). O genoma dos carlavírus é composto por uma molécula RNA de fita simples, senso positivo, possui 6500 a 8600 nt, e poliadenilado (cauda poli A) na região terminal 3', encapsidado por partículas filamentosas flexuosas com dimensões de aproximadamente 610-700 nm de comprimento e 12.15 nm de diâmetro (KING *et al.*, 2011).

A organização típica do gênero *Carlavirus* é de 6 ORF's ("Open Reading Frames" ou fases abertas de leitura) (Figura 1) (King *et al.*, 2011). A ORF1 codifica uma proteína correspondente a replicase, RNA dependente de RMNA polimerase (RdRp); as ORFs 2, 3 e 4 formam as proteínas do TGB (*triplo gene block*) que corresponde a uma região conservada de genes, envolvidas no movimento do vírus célula-a-célula e a longa distância; a ORF 5 codifica a proteína responsável pela proteína capsidial, e a ORF 6 codifica uma proteína com função ainda não determinada (MARTELLI *et al.*, 2007; MENZEL *et al.*, 2010).

Figura 1. Organização genômica dos carlavírus.

Fonte: Adaptado de King et al, 2012.

Para demarcação de espécies, um dos critérios utilizados é a análise dos genes da polimerase ou da proteína capsidial, em que isolados da mesma espécie apresentam identidade de nucleotídeos superior a 70% (ou 80% de identidade de aminoácidos). Vírus pertencentes a diferentes gêneros possuem identidade de nucleotídeos inferior a 45% (KING *et al.*, 2011). Além desses critérios de classificação para uma espécie de carlavírus, a transmissão pelo vetor é importante, pois há espécies de carlavírus transmitidas por afídeos e outras por mosca-branca (King *et al.*, 2011).

No Brasil, o CPMMV foi primeiramente identificado causando mosaico em feijoeiro cv. Jalo (COSTA; GASPAR; VEGA; 1983) e em soja na safra 2000/2001, com plantas exibindo sintomas de nanismo, severa necrose da haste ou até mesmo morte de plantas em grandes áreas do centro oeste do Brasil (Almeida *et al.*, 2003). Nos anos seguintes (2002 e 2003), sintomas de necrose da haste foram relatados nos estados do Maranhão e Paraná e em 2008 a doença foi reportada em diversas regiões produtoras de soja dos estados de Minas Gerais, Goiás, Mato Grosso, Paraná, Bahia, Tocantins e Maranhão (ALMEIDA, 2008). O CPMMV ocasiona em soja sintomas de clorose, mosaico, nanismo, necrose das nervuras e da haste ou até mesmo a morte dependendo do genótipo, de onde provém o nome da doença “necrose da haste” (IWAKI *et al.*, 1982; ALMEIDA *et al.*, 2003, 2005).

Assim como os representantes do gênero, o CPMMV é um vírus de RNA senso positivo de fita simples, possui um genoma de 8200 nucleotídeos com uma estrutura cap [m7GpppG] ligada ao terminal 5' e uma cauda poliadenilada no terminal 3' (KING *et al.*, 2011; ZANARDO *et al.*, 2014a). A gama de hospedeiros inclui espécies

relatadas nas famílias Fabaceae (*Arachis hypogaea*, *Arachis repens*, *Canavalia ensiformis*, *Desmodium tortuosum*, *Glycine max*, *Phaseolus vulgaris*, *Vigna subterranean*, *Vigna unguiculata*), Laminacea (*Salvia hispanica*) e Solanacea (*Solanum lycopersicum*) (ALMEIDA *et al.*, 2003; MENZEL *et al.*, 2010; ZANARDO *et al.*, 2014; CELLI *et al.*, 2016).

Geralmente, os carlavírus são transmitidos por afídeos (KING *et al.*, 2011), porém o CPMMV e o *Melon yellowing-associated virus* (MYaV) são exceções do gênero, pois são transmitidos por *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (COSTA; GASPARG; VEGA, 1983; NAVAS-CASTILLO; FIALLO-OLIVE; SANCHEZ-CAMPOS, 2011). O CPMMV é transmitido por *B. tabaci* de forma não-persistente (MUNIYAPPA; REDDY, 1983; MARUBAYASHI *et al.*, 2010) e também pode ser transmitido por extrato vegetal tamponado. Há relatos de transmissão do CPMMV por sementes em algumas leguminosas como *G. max*, *P. vulgaris* e *V. unguiculata* (THOUVENEL; MONSARRAT, FAUQUET *et al.*, 1882; BRUNT; KENTEN, 1973; HORN *et al.*, 1991; BRITO *et al.*, 2012). A transmissão por semente parece ser dependente do isolado viral, como ocorreu com um isolado de CPMMV de Gana, o qual houve transmissão por sementes em soja, feijão de corda e em feijão comum (BRUNT; KENTEN, 1973), e recentemente foi relatado na Venezuela a transmissão do CPMMV por sementes de *V. unguiculata* subsp. *sesqui pedalis* (BRITO *et al.*, 2012). No Brasil, ainda não há relatos de transmissão do CPMMV por sementes de soja (ALMEIDA *et al.*, 2005).

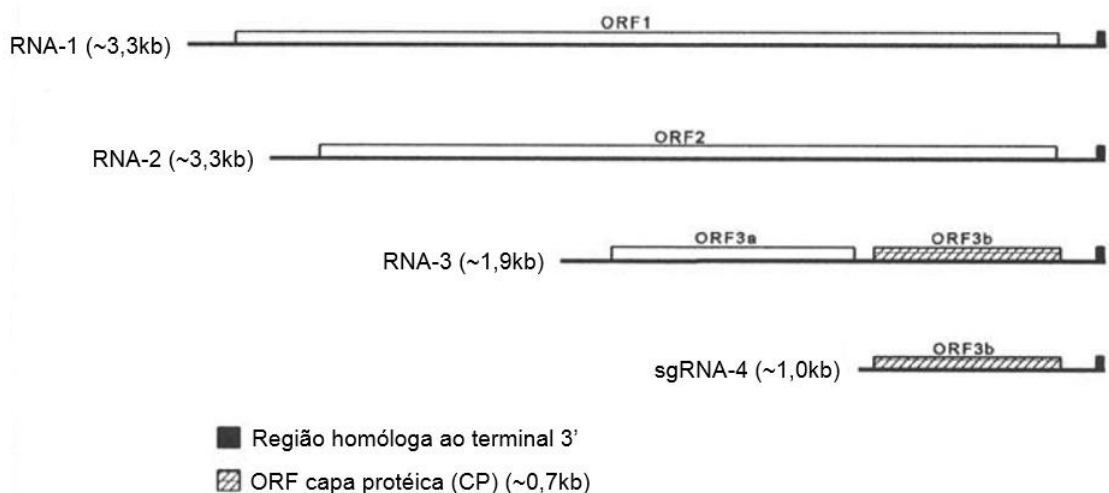
Apesar dos diversos relatos desse vírus, não há dados recentes sobre a incidência do CPMMV nas principais regiões produtoras e os danos ocasionados à cultura.

Gênero Ilarvirus, espécie Tobacco streak virus

O gênero *Ilarvirus* compreende 22 espécies sendo o de maior número dentro da família *Bromoviridae*, que possui 6 gêneros e um total de 36 espécies (ICTV, 2019). Os ilarvirus possuem um genoma tripartido de RNAs de fita simples, senso positivo (ssRNAs) sem a presença de cauda poli A, separados e encapsidados por partículas esféricas de aproximadamente 36 nm de diâmetro (PALLAS *et al.*, 2013; KING *et al.*, 2012). O genoma típico dos ilarvírus possui um comprimento total de aproximadamente 8622 nt. O RNA 1 (~3400 nt) codifica uma proteína da replicase viral. O RNA 2 (~2900 nt) codifica a RNA dependente de RNA polimerase. O RNA 3

(~2200 nt) codifica a proteína de movimento (PM) (PALLAS *et al.*, 2013). A proteína capsidial (CP) é expressada via um RNA 4 subgenômico, colinear com o final 3' do RNA 3 (Figura 2) (BOL, 1999).

Figura 2. Organização genômica dos ilarvírus.



Fonte: Adaptado de Foster e Taylor, 1998.

Os critérios utilizados para a demarcação das espécies no gênero incluem: sorologia, gama de hospedeiros e similaridade das sequências de nucleotídeos, porém as porcentagens de identidade não foram definidas para a diferenciação das espécies (KING *et al.*, 2011).

O *Tobacco streak virus* é o membro tipo do gênero *Ilarvirus* e foi identificado pela primeira vez no Brasil em 1940 em plantas de fumo (*Nicotiana tabaccum* L.) (COSTA, 1945). Em soja, o primeiro relato foi feito em 1955 (COSTA; MIASAKA; D'ANDREA PINTO, 1955), e desde então, sua ocorrência em campos de produção de soja é muito variável estando mais restrita a regiões dos Estados do Paraná e de São Paulo (ALMEIDA *et al.*, 1995), devido a presença natural de hospedeiros do vírus como a cravonara (*Ambrosia polystachya*). O TSV tem como hospedeiros o algodoeiro (*Gossypium hirsutum* L.), tomateiro (*Solanum lycopersicum* L.), amendoim (*Arachis hypogaea* L.), girassol (*Helianthus annuus* L.) (COSTA; CARVALHO, 1961).

Os sintomas de curvatura e necrose do broto da soja são característicos do TSV, e dão origem ao nome da doença “queima do broto da soja” (Almeida *et al.*, 1995). Os sintomas aparecem logo nos estádios iniciais, os trifólios ficam amarelados e as folhas mais velhas apresentam sintomas de mosqueado e mosaico. Em estádios mais avançados as plantas apresentam nanismo e atraso da maturação

(RABEDEAUX *et al.*, 2005; ALMEIDA, 2008). O superbrotamento de vagens e trifólios também é comum em plantas infectadas por TSV (IRIZARRY *et al.*, 2016).

A transmissão do TSV envolve o movimento físico do pólen infectado de uma planta para outra tendo os tripes como vetor. No Brasil, a transmissão do TSV foi associada a presença de insetos do gênero *Frankliniella* (Thysanoptera: Thripidae) (COSTA & LIMA NETO, 1976). Em outros países, a transmissão por espécies de *F. occidentalis*, *F. schultzei*, *Trips tabaci*, *T. parvispinus* e *Microcephalothrips abdominalis* já foi descrita (KAISER *et al.*, 1982; GREBER *et al.*, 1991; KLOSE *et al.*, 1996). O TSV também é transmitido por sementes de soja (GHANEKAR; SCHWENK, 1973).

CAPÍTULO 1 - Effects of the carlavirus Cowpea mild mottle virus on soybean cultivars in Brazil

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Soybean stem necrosis is caused by *Cowpea mild mottle virus* (CPMMV), transmitted by the whitefly *Bemisia tabaci*. CPMMV has already been recorded in all major soybean-producing areas of Brazil. The impacts caused by CPMMV to Brazilian soybean production are unknown. Thus, the goal of this study was to evaluate the effects of CPMMV on the major soybean cultivars grown in the Southern and Midwestern regions of Brazil. Although asymptomatic in some of the tested cultivars, CPMMV infection significantly reduced the plant height, the number of pods per plant and the 1,000-grain weight. In addition, estimated yield losses ranged from 174 to 642 kg ha⁻¹, depending on the cultivar. Evidence of seed transmission of CPMMV was observed in the BMX POTENCIA RR cultivar. These results suggest that CPMMV could have an important role in the reduction of soybean productivity in Brazil. Symptomless infections might be hiding the actual impact of this pathogen in

commercial fields and infected seed could be the primary inoculum source of the virus in the field.

Keywords: CPMMV; stem necrosis; soybean disease; transmission

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important crop worldwide as a source of oilseed and protein. Brazil is the second largest producer of soybean in the world, producing 119.23 million tons in a cultivated area of 35.1 million hectares in the last season (2017/2018). In Brazil, soybean is the most important economic crop, generating approximately 675 million US\$ to the internal market and 31 billion US\$ to exportation (Hirakuri & Lazzarotto, 2014; Conab, 2018). In the last decade in Brazil, the cultivated area and production increased 64.9% and 98.7%, respectively, between the 2007/2008 and 2017/2018 crop seasons, while the productivity increased from 2,800 to 3,400 kg ha⁻¹ over the same time (Conab 2018).

Soybean can be affected by several pests regardless of growth stage. In Brazil, the occurrence of at least 15 viral diseases have been reported (Almeida 2008; De Marchi et al. 2018). The *Cowpea mild mottle virus* (CPMMV; family *Betaflexiviridae*, genus *Carlavirus*), which is the agent of soybean “stem necrosis disease”, was reported infecting soybean in the 2000/2001 season in the state of Goias. CPMMV is considered an emergent virus and has been already recorded in soybean fields across Brazil in the states of Bahia, Mato Grosso, Maranhao, and Parana (Almeida et al. 2003; Almeida 2008; Almeida et al. 2005). In 2008, the virus was observed in the Minas Gerais and Tocantins states, which are important soybean growing areas (Almeida 2008). Although steam necrosis is the common name of this disease, in the last years, mild mottle and mosaic were the most common symptoms observed for this virus infection

(Zanardo et al. 2014; Zanardo e Carvalho 2017). Thus, the symptoms of this disease do not match with its common name, what brought us the interest to study this pathosystem.

In general, carlaviruses are transmitted by aphids (King *et al.*, 2011). However, CPMMV is one of the two exceptions of the genus that are transmitted in a non-persistent manner by whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Almeida 2008; Marubayashi et al. 2010), pest that has been an important factor in the reduction in soybean production (Lourenção et al., 1999; Tamai et al., 2006) and causing losses to several crops such as vegetables, fibres and ornamentals (De Barro et al. 2011; Lapidot et al. 2014) by virtue of its ability to transmit numerous viruses (Navas-Castillo et al. 2011). *Bemisia tabaci* is widely spread in Brazil, and the Middle East Asia Minor 1 (MEAM1, known as B biotype) is the prevalent species on major crops across the country, and it is mainly associated with CPMMV infection in soybean (Moraes et al. 2018). The species Mediterranean (MED, also known as biotype Q), which was recently related in the country and it is distributed in the southeast and south region, is associated with insecticide resistance which has been caused its rapid invasion and dominance in new niches and countries (Moraes et al. 2018; Horowitz e Ishaaya 2014; Yao et al. 2017). Currently, *B. tabaci* is between the ten most important pest in Brazil (Brasil 2019).

CPMMV is also easily transmitted by sap (Brunt e Kenten 1973), one characteristic that in artificial conditions is very helpful to study the virus. The seed transmission of CPMMV has also been reported in different plant species such as soybean, cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*) in Africa (Brunt e Kenten 1973), in addition to yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis*) seed in Venezuela (Brito et al. 2012), and by some soybean cultivars in India (Yadav et al.

2013). To date, there have been no reports of CPMMV isolates transmitted by soybean seed in Brazil (Almeida et al. 2005).

The effects caused by CPMMV to Brazilian soybean production have never been estimated (Zanardo e Carvalho 2017), and the ability of Brazilian CPMMV to be transmitted by seed is still unknown. Thus, the goal of this study was to evaluate the damage of CPMMV on the major soybean varieties used in the main growing areas of the Southern and Midwestern regions of Brazil. Additionally, the seed transmission ability of one CPMMV isolate from the São Paulo State was assessed.

MATERIAL AND METHODS

Obtaining CPMMV isolate and partial genome characterisation

The CPMMV isolate was collected from soybeans in Casa Branca County, São Paulo State, Brazil (2016/2017). Total RNA was extracted from the leaf tissue of symptomatic soybean plants using the RNA Purification Kit (NORGEN) followed by reverse transcription-polymerase chain reaction (RT-PCR) One Step using AMV reverse transcriptase (Promega, Brazil). The specific primers were CPMMV 1280-F (5'-GGC GTT CCA AAA GCT GCC GAT-3') and CPMMV 1696-R (5'-GGA GCC ACC TTT CCA ATC AA-3') (De Marchi et al. 2017). All amplifications consisted of an initial step of 42°C for 30 min, a second step of 94°C for 2 min, 30 cycles of 94°C for 54 sec, annealing at 54°C for 50 sec and elongation at 72°C for 50 sec, followed by a final extension step at 72°C for 10 min. In order to obtain the partial genome characterisation of the CPMMV isolate of Casa Branca – SP (called CPMMV-Casa Branca), the ORF 2 to the 3' terminus was amplified with a RT-PCR using the following four primer pairs: 5,722 F (5'-ATG GGG GAA CGA GTA AC TGC-3') and 6,528 R (5'-TCA CTG TGC CTG GTC AA ACA-3'); 6,240 F (5'-ACA GGT TCG GTA AGA GC

ACG-3') and 7,173 R (5'-GCT TTT GGA ACG CCA GT TTT-3'); 7,152 F (5'-CAA AAA CTG GCG TTC CAA A AGC-3') and 8,003 R (5'-AGG GAA ACG TGA ATG GC GAT-3'); 7,921 F (3'-AGG GCC TGA AAT ATC TCG CA-3') and 8,322 R (3'-GGA CTC TTT ATA CTC TTT CCT T GGT-3'). The amplifications consisted of an initial step of 42°C for 30 min, second step of 95°C for 2 min, 35 cycles of 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The amplified PCR products were purified (QIAquick Gel Extraction Kit, Qiagen) and directly sequenced in both forward and reverse directions. The sequence obtained was analysed using the software Geneious v9.1.3. (Kearse et al. 2012) and compared to different CPMMV isolates described by Zanardo *et al.*, (2014).

After virus identification and characterisation, the isolate was maintained in common bean (*Phaseolus vulgaris* L.) cv. Jalo by whitefly transmission. Virus transmission was performed by transferring whitefly specimens (MEAM1) in cages containing infected soybean leaves for a viral acquisition access period (AAP) of 24 h. Following virus acquisition, whiteflies were transferred to cages containing healthy bean plants at the VC (cotyledon leaves) growth stage, for a 24-h inoculation access period (IAP) under controlled conditions at 30°C. After inoculation, insecticides (Oberon and Cartap) were sprayed on plants to eliminate all the whitefly adults, nymphs and eggs. Thirty days after the IAP, plants were analysed for the virus presence. After virus confirmation, the plants were used as source of inoculum for the field inoculation.

Experimental areas

Field experiments were conducted during the 2017/2018 growing season with six cultivars distributed in four different growing areas: cv. BMX POTENCIA RR in Botucatu, State of São Paulo (coordinates: 22°48'25.4"S, 48°25'46.4"W, elevation: 739 m, sowing date 01/11/2017), cv. M 6410 IPRO and TMG 7062 IPRO in Mogi Mirim,

State of São Paulo (coordinates: 22°26'42.8"S 47°04'10.8"W, elevation: 687 m, sowing date 15/12/2017) , cv. M 7739 IPRO and M 8372 IPRO in Pedra Preta, State of Mato Grosso (coordinates: 16°50'30.3"S 54°02'39.8"W, elevation: 744 m, sowing date 23/11/2017), and cv. M 9144 RR in Planaltina, Federal District (coordinates: 15°39'51.7"S 47°20'02.0"W, elevation: 887 m, sowing date 27/11/2017).

The four sites represented some of the major soybean producing areas in Brazil. They have contrasting environmental conditions, and the cultivars were selected according to the frequency that they were planted in each region. Parameters such as the occurrence of weeds, diseases and insect pressure, especially *B. tabaci*, were monitored throughout the season.

Experimental design and field inoculation

The experimental design was a randomised block, with two treatments (plants infected by sap transmission and healthy plants) with five replications. The plot was comprised of six rows (5m), 0.45 m between rows and an average of 14 plants per meter.

For the field inoculation, the virus was readily sap-transmitted in soybean plants 30 d after sowing using infected leaves ground in phosphate buffer 0.01 M, pH 7, using approximately two hundred soybean plants per plot. The presence of the virus was detected by RT-PCR using primers specific for CPMMV.

Field sampling and evaluation of agronomic traits

A total of 100 soybean samples collected in each plot were evaluated for virus infection 30 days after inoculation. When the plants were at physiological maturity (R8), the plant height was evaluated by the distance from the soil to the apex of the plant (cm) and the number of pods per plants was obtained by counting the total number of pods per plant. At the harvest, all plants of the plot were hand harvested and run through a

thresher. For each plot, the 1,000-grain weight (g) was determined, which was obtained by weighing 1,000 grains from the plants in the plot, and adjusting to 13% moisture in addition to grain productivity, which was obtained by weighing the grains produced, and adjusting to 13% moisture, then converting into kg ha⁻¹.

Soybean seed transmission

To study the seed-borne capacity of CPMMV, a random sample of seed was collected from the CPMMV-infected BMX POTENCIA RR plot, harvested in the Botucatu field. These soybean seeds were planted in Styrofoam seedling trays containing Tropstrato HA Hortaliças (Vida Verde Tecnologia em Substratos, Mogi Mirim, SP). The seedling trays were kept in an insect-proof cage. Germination was greater than 90%, and the seedlings did not show any typical disease symptoms. For virus detection, leaf samples were tested using RT-PCR. To compose a sample, leaves of ten plants were collected and combined. There were 80 samples tested, totalling 800 plants. Once the presence of CPMMV was detected in a sample, the ten plants were tested individually to identify the infected plant. The positive plants were kept in an insect-proof cage for 60 days in order to observe the symptoms.

Data Analysis

Because of the interdependency and interrelationship of agronomic traits, principal component analysis (PCA) was performed to investigate the data collected in the current study. PCA was performed using Minitab 17 Statistical Software (2010). Data were also submitted to analysis of variance (ANOVA) using Statview software (Concepts and StatView, 1987) to determine whether significant differences ($p < 0.05$) occurred between treatments. Then means were compared using Tukey's test ($\alpha = 5\%$).

RESULTS

Virus characterisation

The partial genome of the CPMMV-Casa Banca isolate (ORF 2 to the 3' terminus) (Accession number MK900736) obtained in this study showed 99% of nucleotide identity with the CPMMV:BR:MT:02:1 and Florida_Bean_2011 isolates retrieved from GenBank (Accession numbers: KC884246.1 and KC774020.1, respectively). According to the classification used by Zanardo *et al.* (2014), the CPMMV-Casa Branca isolate belongs to the BR2 group, which encompasses the most common CPMMV strains from soybean in Brazil. This isolate was used in all field experiments.

Virus incidence and symptoms on inoculated plots

The symptoms observed were variable among the cultivars. BMX POTÊNCIA RR, M 7739 IPRO and M 8372 IPRO cultivars were symptomless to CPMMV infection. In contrast, the M 6410 IPRO, TMG 7062 IPRO and M 9144 RR cultivars showed the most severe symptoms. The most common symptoms were chlorosis, mottling and mild symptoms (Figure 1). Due to the variation in symptoms, the estimate of virus infection was carried out by sampling the plots followed by molecular analysis. All the six sap-inoculated soybean cultivars had an infection from the CPMMV-Casa Branca isolate greater than 70% in field conditions (Table 1). The virus transmission efficiency was 70% in cv. M 8372 IPRO, 85% in cv. BMX POTÊNCIA RR, 87% in cv. M 9144 RR, 89% in cv. M 7739 IPRO, 90% in cv. TMG 7062 IPRO and 92% cv. M 6410 IPRO.

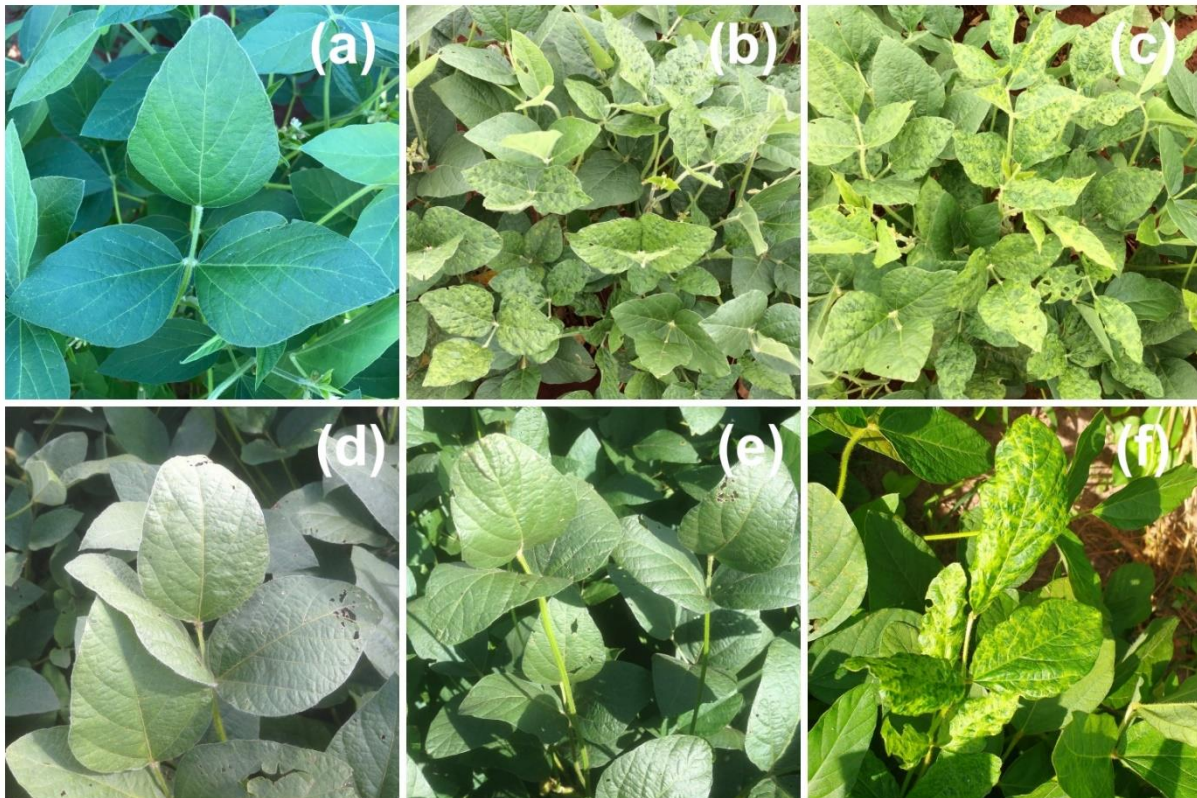


Figure 1. Symptoms observed in the six cultivars tested in the field experiment: (a) BMX POTÊNCIA RR, (d) M 7739 IPRO and (e) M 8372 IPRO showed to be symptomless; (c) M 7062 IPRO, (d) M 8372 IPRO and (f) M 9144 RR showed chlorosis, mild and mottling symptoms

Field plot experiments and agronomic performance of the cultivars

The PCA of soybean cultivars comparing CPMMV-infected and healthy plants showed the proportion of the variance retained by the first principal component (PC1) was 67.5% and for the second principal component (PC2) corresponded to 22.3% of the original remaining variance (Figure 2).

The exploratory analysis allowed the evaluation of the virus influence on the groups of cultivars and cultivated areas (Figures 2 and 3). Among the cultivars, the cv. M 9144 RR healthy plants had the greatest plant height and were among the varieties that have the highest number of pods per plants, which set this cultivar and treatment apart from the rest. Regarding the 1,000-grain weight, the cultivar that showed the best

performance was TMG 7062 IPRO followed by M 7739 IPRO, M 6410 IPRO and BMX POTÊNCIA IPRO. The greatest productivity was reached by the cultivar BMX POTÊNCIA IPRO, followed by M 7739 IPRO and M 8372 IPRO, being the cv. BMX POTÊNCIA the most affected by the presence of the virus.

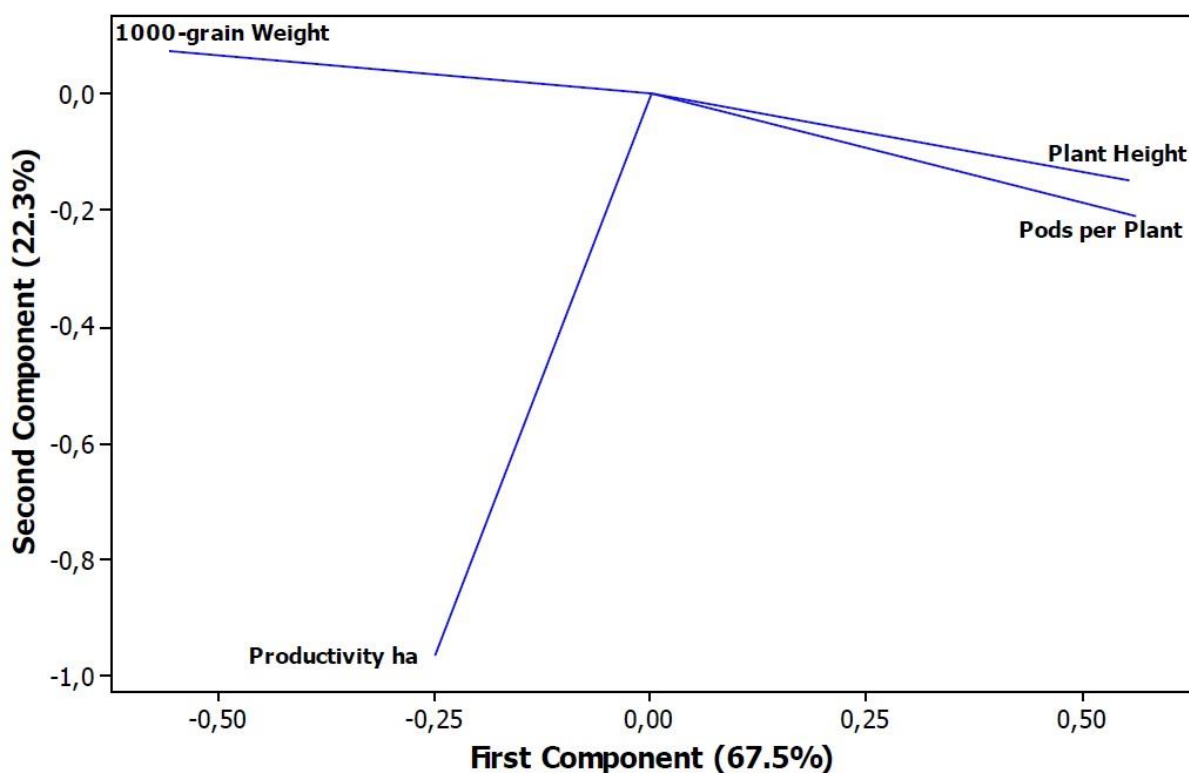


Figure 2. Projection of vectors of traits: plant height in cm, number of pods per plants, 1000-grain weight in g and productivity in bags ha^{-1} .

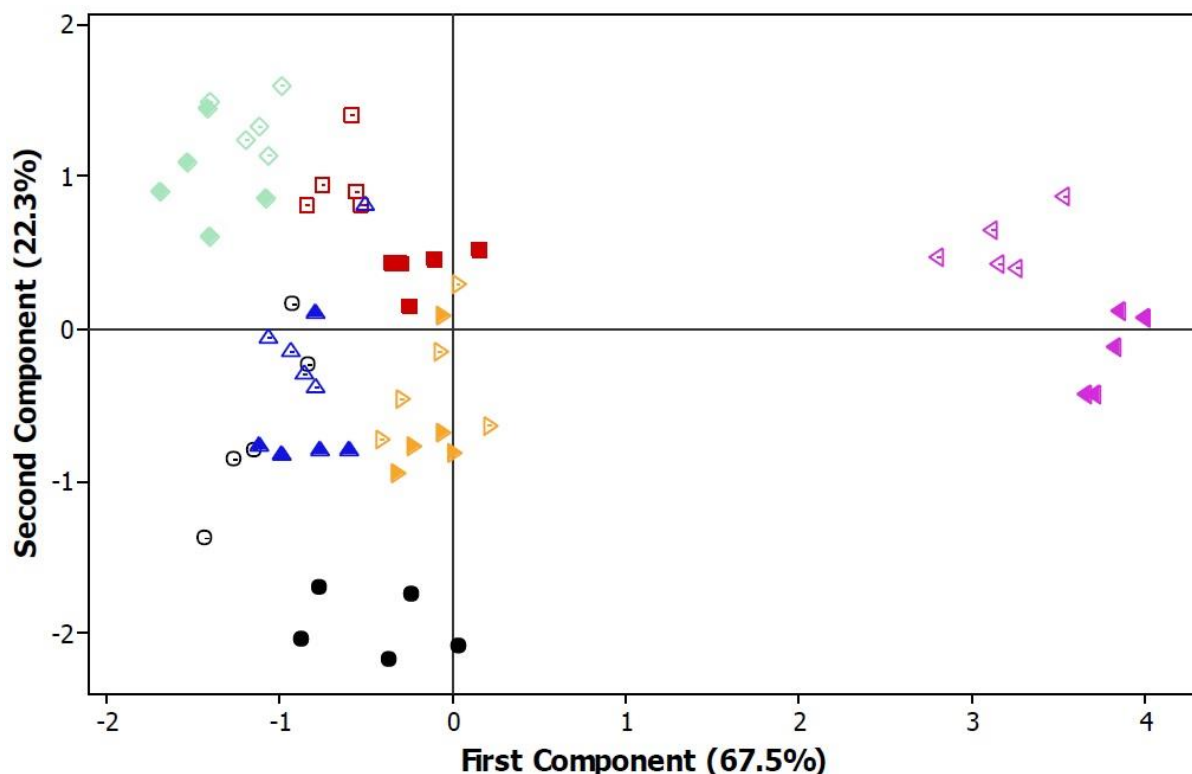


Figure 3. Biplot graph with dispersion of six soybean cultivars according to the principal components (PC1 and PC2). ● cv. BMX POTÊNCIA RR healthy plants, ○ cv. BMX POTÊNCIA RR CPMMV-Infected plants, ■ cv. M 6410 IPRO healthy plants, □ cv. M 6410 IPRO CPMMV-Infected plants, ◆ TMG 7062 IPRO healthy plants, ◇ TMG 7062 IPRO CPMMV-Infected plants, ▲ cv. M 7739 IPRO healthy plants, △ cv. M 7739 IPRO CPMMV-infected plants, ► cv. M 8372 IPRO healthy plants, > cv. M 8372 IPRO CPMMV-infected plants, ◄ cv. M 9144 RR healthy plants, < cv. M 9144 RR CPMMV-infected plants.

As expected, each cultivar had distinct performance once they have particular characteristics and they were cultivated in areas with contrasting environmental conditions (Figure 3). The exploration of the data also demonstrated that the cultivars M 6410 IPRO and TMG 7062 IPRO cultivated in Mogi Mirim - SP had a close performance to all evaluated traits as well as the cultivars M 7739 IPRO and M 8372 IPRO which were cultivated in Pedra Preta – MT. The cv. M 9144 RR tested in Planaltina – DF showed the most distant performance data comparing to the other cultivars (Figure 4).

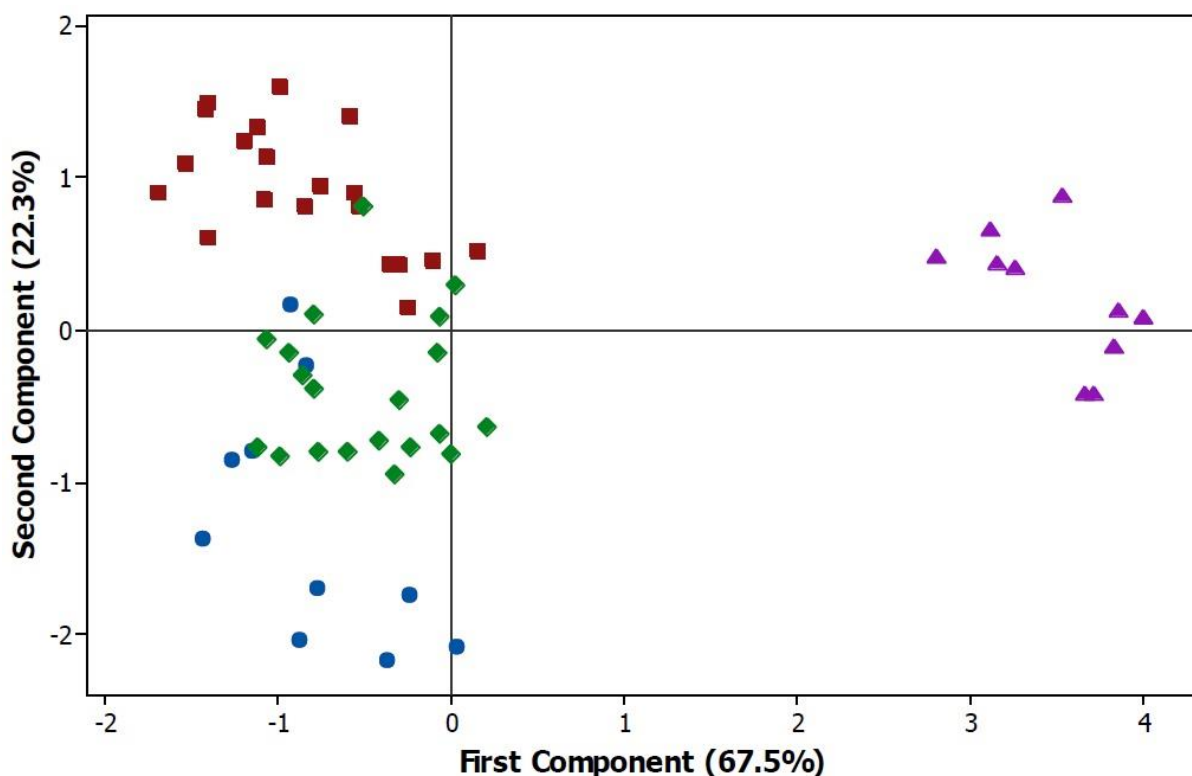


Figure 4. Biplot graph with dispersion of four areas according to the principal components (PC1 and PC2). ● Botucatu, São Paulo, ■ Mogi Mirim, São Paulo, ◆ Pedra Preta, Mato ▲ Planaltina, Federal District.

Analysis of variance showed that cultivar tested in this work presented different response to the virus infection, showing a significant ($p < 0.05$) or non-significant reduction in the traits evaluated (Table 1).

There was a significant effect ($p < 0.05$) of CPMMV-infection in all agronomic traits evaluated in the cv. BMX POTÊNCIA RR. CPMMV-infected plants have reduction in plant height ($p < 0.01$, $F = 467.76$), number of pods per plant ($p < 0.01$, $F = 36.53$) and 1,000-grain weight ($p < 0.01$, $F = 11.64$) that reflected directly in the productivity ($p = 0.01$, $F = 11.20$), which had a loss of 638 kg ha^{-1} , this cultivar being the one that had the greater yield loss, approximately 16%.

Table 1. Percentage of infected plants and mean of agronomic traits of fields assays according to their cultivar.

Location	Cultivar	Infected Plants (%)	Plant Height (cm)		Pods per plant		1,000-grain weight (g)		Yield (kg ha ⁻¹)	
			Healthy	CPMMV-infected	Healthy	CPMMV-infected	Healthy	CPMMV-infected	Healthy	CPMMV-infected
Botucatu	BMX POTENCIA RR	85	78.80a	60.60b	82.10a	40.30b	149.60a	132.60b	4,029a	3,391b
Mogi Mirim	M 6410 IPRO	92	68.71a	59.71b	70.15a	41.26b	139.37a	129.13b	2,564a	2,330b
	TMG 7062 IPRO	90	67.13a	66.86a	34.22a	30.92b	186.05a	169.01b	2,393a	2,145b
Pedra Preta	M 7739 IPRO	89	74.76a	70.84b	45.68a	42.89a	145.54a	144.97a	3,323a	2,953a
	M 8372 IPRO	70	86.84a	84.48a	50.82a	44.40b	123.66a	119.00a	3,230a	3,056a
Planaltina	M 9144 RR	87	123.13a	121.07a	61.60a	42.45b	148.71a	105.45b	2,278a	1,962b

Mean followed by the same letter within rows indicate no significant (p < 0.05) difference between healthy and CPMMV-infected plants according to an ANOVA.

Regarding the cultivar M 6410 IPRO, there was also a significant reduction ($p < 0.05$) in all traits evaluated, and only the plant height ($p=0.74$, $F=0.1$) did not differ to the cv. TMG 7062 IPRO. The cultivars tested in Rondonopolis differed significantly only in one parameter each, plant height ($p<0.01$, $F=17.92$) and number of pods per plant ($p<0.01$, $F=8.97$), for cv. M 7739 IPRO and M 8372 IPRO, respectively. Although no significant effect of treatment in the yield occurred in both cultivars, there was a reduction of 370 kg ha⁻¹ for cv. M 7739 IPRO ($p=0.07$, $F=4.08$), and 174 kg ha⁻¹ for cv. M 8372 IPRO ($p=0.33$, $F=1.07$).

All traits in the cv. M 9144 RR, except for plant height, were affected significantly ($p<0.05$) due to CPMMV-infection. Although the height was significantly the same for the treatments ($p=0.51$, $F=0.47$), the reduction caused in number of pods per plant ($p=0.25$, $F=7.57$) and 1000-grain weight ($p<0.01$, $F=182328.14$) in the diseased plants directly affected the in productivity ($p=0.01$, $F=9.85$) with a reduction of 316 kg ha⁻¹, or approximately 14%.

Seed transmission

From 800 seedlings obtained from seeds harvested in the cv. BMX POTÊNCIA RR CPMMV-infected field plot, three plants were found to be infected by CPMMV. The RT-PCR confirmed the presence of the systemic infection of BMX POTÊNCIA RR plants coming from seeds collected from CPMMV-infected soybean plants. Not a single plant developed typical disease symptoms after emergence until their senescence. The observed percentage of plants infected with the virus was 0.375%.

DISCUSSION

The data obtained in this study revealed that CPMMV causes reduction of agronomic traits such as productivity, plant height, 1000-grain weight and pods per plant in the

main soybean cultivars used in Brazil, suggesting that this virus may be responsible for major economic losses for soybean crop in our country. Additionally, the CPMMV-seed transmission data for a Brazilian isolate highlights the seed importance as a primary inoculum source in the field, especially in areas with low whitefly population, such as in Parana south region, Santa Catarina and Rio Grande do Sul States.

CPMMV was first recorded in Brazil in common bean in the 1980s (Costa *et al.*, 1983), and reported as a threat to soybean production in 2002 (Almeida *et al.* 2003). The losses reported at that time were higher than 85%, since the cultivars used were developing the stem necrosis symptom that affected the whole plant (Almeida *et al.* 2003). The new cultivars has reduced the impact of this disease in soybean (Arias *et al.* 2015). However, although some soybean cultivars are symptomless when infected by CPMMV, they can have great yield losses, as our results suggest.

In addition, *B. tabaci* MEAM1 is the predominant species in the country, and MED is distributed in the southeast and south region (Moraes *et al.* 2018). More recently, MED has reported in the middle-western region (data not published). The Brazilian middle-western region is the largest soybean and common bean producer, and both crops are cultivated near to each other. It is already known that MEAM1 can colonize soybean and common bean as well as transmit viruses (Marubayashi *et al.* 2010; Inoue-Nagata *et al.* 2016), but recent studies revealed that MED has a great reproduction in both crops, especially common bean (Watanabe *et al.* 2019) and is a good vector of many Brazilian viruses (Bello *et al.* 2019). Then, the combination of these conditions may help the CPMMV to spread, to cause damage in plants and to be present in the field during the whole year. Currently, the virus management is based on the chemical control of *B. tabaci* (Yao *et al.* 2017). However, another important parameter that should be included in the management is the Brazilian agriculture system, which is

complex and has contributed to increase the whitefly population as well as the virus dissemination (Inoue-Nagata et al. 2016). In addition, as soybean and common bean are grown in nearby areas, common bean can also serve as inoculum source to soybean crop, contributing to spread the virus and to reduce the soybean yield. In our study, the highest reduction in productivity was observed for cv. BMX POTENCIA RR (638 kg ha⁻¹) followed by cv. M 7739 IPRO (370 kg ha⁻¹) and M 9144 RR (316 kg ha⁻¹) (Table 1). It is also important to highlight that even a reduction in productivity of 174 kg ha⁻¹ observed for cv. M 8372 IPRO may cause a huge economic impact, since the bag (60 kg) price is, on average, 20.00 US\$ (CEPEA, 2019). As Brazil is the largest soybean oilseed exporter in the world (USDA. 2019), the amount of bags reduction impacts directly not only for the farmers, but also for the Brazilian economy.

Seed-borne transmission has already been reported for CPMMV isolates worldwide, but here we provide the first evidence of seed-transmission of a CPMMV Brazilian isolate in soybean. Seed transmission was observed in cowpea, soybean and common bean seeds in Ghana, even though at a low incidence (Brunt e Kenten 1973). In Thailand, the virus was also observed to be transmitted by soybean seeds at a frequency lower than 1% (Iwaki *et al.*, 1982). In Venezuela seed transmission was observed for yardlong bean seeds (Brito et al. 2012). In India, the seed-borne nature of the virus was detected in several soybean cultivars with higher rates of transmission, ranging from 0.62% to 14.2% (Yadav et al. 2013). The seed transmission ability for CPMMV seems to be dependent on the viral isolate and the host cultivar and is an important component of the epidemiology of the virus in the field. A few infected seeds can provide enough CPMMV inoculum to be disseminated by the efficient *B. tabaci* vector. Then, the virus can be easily disseminated in the cultivated area, making its management even harder in tropical countries. In the world scale, the CPMMV isolates

from Florida, Puerto Rico and Brazil have shown to be phylogenetically close, indicating a common origin (Zanardo & Carvalho, 2017). Our data reinforce that the CPMMV capacity to be transmitted by seeds might have disseminated the virus in different countries through the international trade.

In summary, soybean productivity was decreased by CPMMV infection and losses of more than 637.8 kg ha⁻¹ were observed for the symptomless cv. BMX POTENCIA RR, one of the most popular cultivars used in Brazil. Also, the Brazilian agriculture system helps the CPMMV and the whitefly to be in the field during the whole year as well as to be disseminated, increasing the concern about this disease. Seed transmission of CPMMV was also observed for this cultivar, even in low frequency. CPMMV is a serious concern for soybean production in Brazil and it cannot be underestimated as it has been in the last years. Thus, because of these conditions, it is important to improve the management, including whiteflies populations, soybean cultivars and seed analysis in order to avoid the CPMMV spread. Then, the amount of losses might be reduced. In addition, there is a need for implementing CPMMV in soybean breeding programs in order to identify varieties with higher tolerances to this virus.

CONCLUSIONS

Soybean productivity was decreased by CPMMV infection and losses of more than 637.8 kg ha⁻¹ were observed for the symptomless cv. BMX POTENCIA RR, one of the most popular cultivars used in Brazil. Seed transmission of CPMMV was also observed for this cultivar, even in low frequency.

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CAPÍTULO 2 - Natural infection of soybean plants by tospovirus, carlavirus and ilarvirus in Brazil

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Abstract

Soybean is a major food crop in Brazil and the primary source of protein and vegetable oil worldwide. Although during the last decades soybean production has increased through development of new varieties, this crop hosts a wide range of pathogens that causes significant yield losses. Among pathogens that affects soybean, the viruses have been drawing attention due to their diversity of symptoms and the

lack of information about their actual impacts in soybean in Brazil. In this work, we have detected and identified three viruses in a commercial soybean field in the County of Mogi Mirim in the State of Sao Paulo by high throughput sequencing analysis. Sequences of a *Carlavirus*, *Tospovirus* and *Ilarvirus* in a pooled sample of 10 plants suggests a mix infection of *Tobacco streak virus* (TSV) and *Cowpea mild mottle virus* (CPMMV), and CPMMV and *Groundnut ringspot virus* (GRSV) and reinforce the importance of whiteflies and thrips as vector of viruses in soybean. Based on the complete genome sequence analysis of the TSV isolate obtained in this study we conclude that our isolate is phylogenetic close to a TSV isolate from Australia.

Keywords: TSV, CPMMV, GRSV, soybean disease, bud blight

Soybean [*Glycine max* (L.) Merrill] is a major food crop in Brazil and its production has been improved significantly in the past decades (Conab, 2018). Although the Brazilian soybean production is in evidence, growers of this leguminous face the raise of several diseases, and among the pathogens affecting this crop, viral diseases has an important role as a reducing factor (Almeida 2008).

Until the beginning of the 2000s, about fourteen diseases in soybean associated with virus were reported in Brazil (Almeida 2008). During the last two decades other virus affecting soybean has been identified in Brazil as the tospovirus *Groundnut ringspot virus* (GRSV) (DeMarchi et al., 2018; Fontes et al., 2019), as well as other known diseases has re-emerged like the carlavirus *Cowpea mild mottle virus* (CPMMV) (Barreto da Silva et al., Unpublished Data).

In the 2018/2019 season, a commercial soybean field in Mogi Mirim County, State of São Paulo, began to draw attention since plants showed severe stunting, reduced leaf size and delayed maturity. High population of thrips was also associated to the plants. To investigate the occurrence of viruses in this field, a pooled sample of ten leaves, collected from individual plants, was characterized by high throughput sequence analysis. Total RNA extraction was performed for the pooled sample with the PureLink Viral RNA/DNA kit (Invitrogen), followed by library preparation and transcriptome sequencing using the Illumina HiSeq2500 platform. *De novo* assembly of the 30,636,554 reads was carried out using the CLC Genomics Workbench software v7.0.3. The 13,345 contigs obtained were submitted to a BLASTn search using the Geneious software v9.1.5.

RNA seq analysis revealed the presence of the carlavirus CPMMV, the tospovirus GRSV and the ilarvirus *Tobacco streak virus* (TSV) in the pooled sample. RT-PCR using tospovirus universal primers BR60/BR65 (Eiras et al. 2001), the carlavirus CPMMV specific primers CPMMV 1280-F/CPMMV 1696-R (De Marchi et al. 2017) and the designed primers TSV ORF2a 899 F (CAC AGG GTC CCA ACT CAG C) and ORF2a 1200 R (ACT GGC TTC AAG GTG GTC TTT) was performed for the 10 plants separately. The presence of CPMMV was confirmed for all the plants tested. The GRSV was present in one plant and the TSV was identified in seven plants. The presence of CPMMV and GRSV was also confirmed for soybean plants collected in nearby fields to Mogi Mirim.

Plants infected with TSV and CPMMV showed reduced plant height, excessive axillary branching, reduced leaf size and mottling symptoms while the plants infected solely with CPMMV presented mosaic symptoms but reduction of growth was not

observed. Plant infected with GRSV + CPMMV presented necrotic areas on leaves, which was not observed for plants infected solely by CPMMV. (Figure 1).

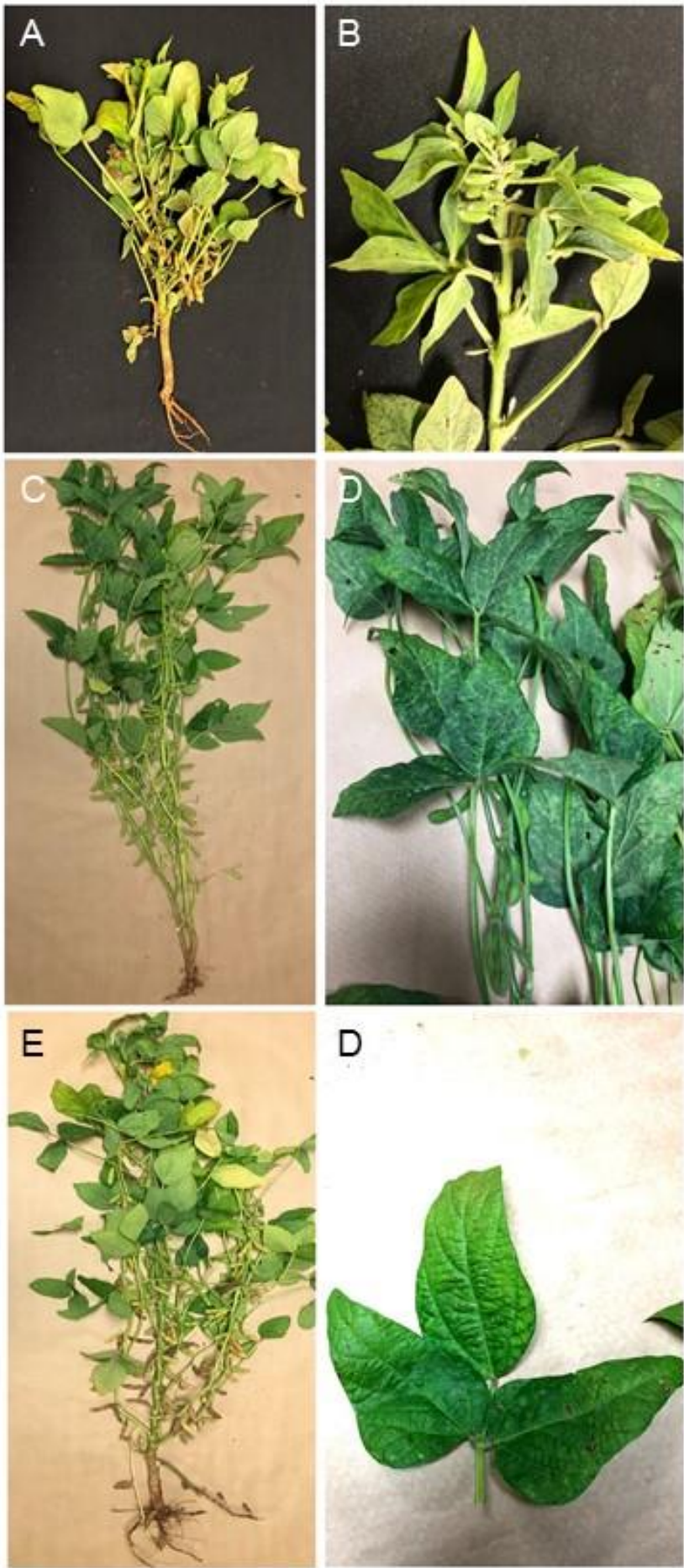


Figure 1. Symptoms observed for: A and B, Soybean plant mix infected with *Cowpea mild mottle virus* and *Tobacco streak virus* exhibiting symptoms of (A) stunt, (B) leaf size reduction and excessive axillary branching; (C and D) soybean plant infected with *Cowpea mild mottle virus* exhibiting symptoms mosaic; and (E and F) soybean plant infected *Cowpea mild mottle virus* and *Groundnut ringspot virus* exhibiting necrotic spots.

The tospovirus was recently reported infecting soybean in the state of Bahia and Federal District, Brazil (De Marchi et al. 2018; Fontes et al. 2019). The CPMMV was detected on soybean in the 2000-2001 growing season (Almeida et al. 2003) and it seems to be an emergent virus in this crop with a variability of isolates detected in Brazil (Zanardo et al. 2014a, b). A recent study also reinforced that CPMMV can be a silent virus in soybean, since cultivars have not shown evident symptoms of the disease, but production can be significantly affected (Barreto da Silva et al., in press).

TSV was first detected in Brazil in 1940 infecting tobacco (*Nicotiana tabacum* L.) (Costa, 1945) and soybean in 1955 (Costa et al., 1955). Since then, TSV incidence varies in functions of the year and the region, and its occurrence is more restrict to the states of Sao Paulo and Parana. TSV infects also cotton (*Gossypium hirsutum* L.), tomato (*Solanum lycopersicum* L.), peanut (*Arachis hypogaea* L.), sunflower (*Helianthus annuus* L.) and some weeds, such as *Ambrosia poystachia* that is a known host of the virus and serves as a source of inoculum in the field (Costa e Carvalho 1961). TSV belongs to the *Ilarvirus* genus and is transmitted mechanically by the thrips feeding on pollen grains containing the virus. Viruses are present in/on the pollen and may be transmitted when wind-blown pollen and populations of vectors thrips are coincident on a susceptible host (Sdoodee e Teakle 1987; Klose et al. 1996; King et al. 2011). Five thrips species can be involved in the transmission of TSV: *Frankliniella*

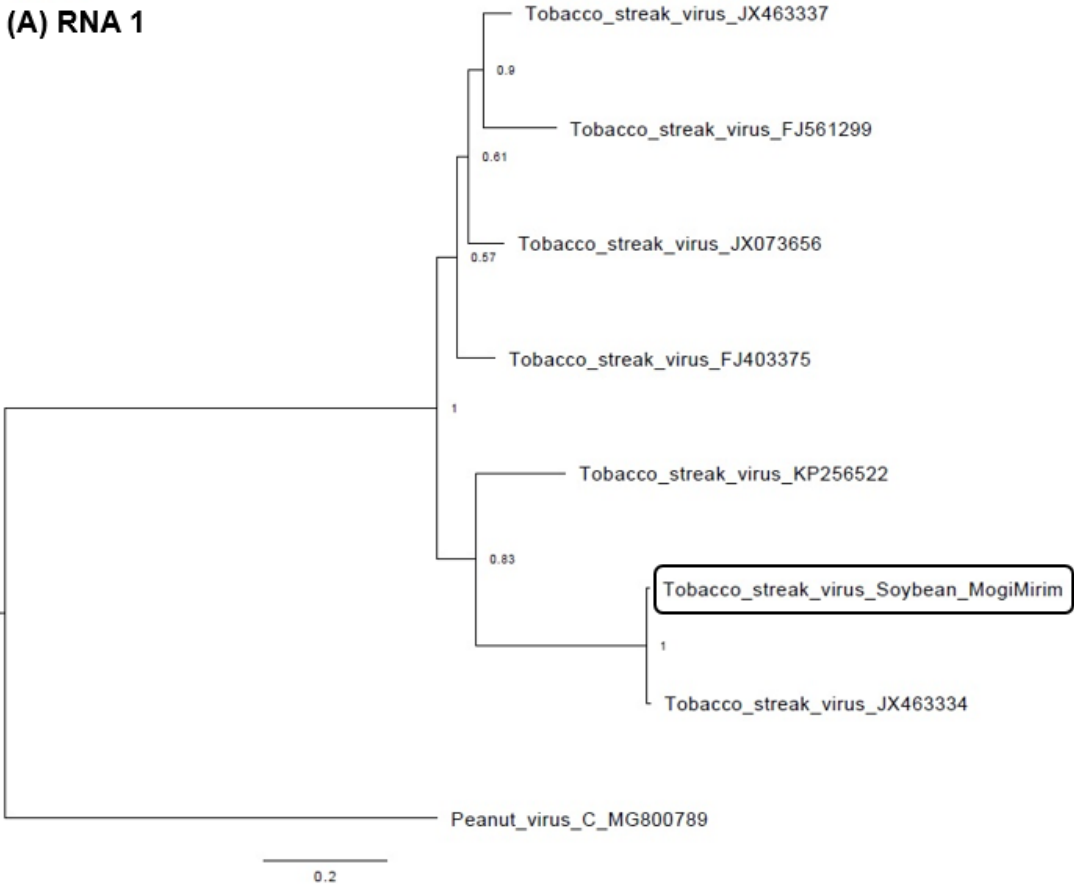
occidentalis, *F. schultzei*, *Trips tabaci*, *T. parvispinus* and *Microcephalothrips abdominalis* (KAISER *et al.*, 1982; GREBER *et al.*, 1991; KLOSE *et al.*, 1996). In Brazil it was demonstrated the association of TSV with the presence of *Frankliniella schulzei* (Costa & Lima Neto, 1976; Moscardi & Almeida, 1980). Seed transmission was also reported in soybean (Ghanekar e Schwenk 1973) and in others host species (Johnson *et al.*, 1984). The disease associated to TSV in soybean plants is characterized as bud blight of soybean, also referred as Brazilian bud blight that causes symptoms of chlorosis, necrosis if leaves, stem, and buds, read node, stunting, leaf size reduction, mosaic, mottle, rugosity and delay of maturation.

Here we obtained the complete genome of the Brazilian soybean isolate of TSV that is composed of a positive-sense single-stranded RNA tripartite genomes (RNA 1 to 3) with a total length of 8622 nucleotides (nt) (King *et al.* 2011). TSV is the type species of the genus *Ilarvirus*, family *Bromoviridae*. RNA 1 (~3400 nt) encode for the viral replication protein and the RNA 2 (~2900 nt) is coding for RNA-dependent RNA polymerase (RdRp). RNA 3 (~2200 nt) code for the movement protein (MP) (King *et al.* 2011; Pallas *et al.* 2013). The viral coat protein (CP) is expressed via a subgenomic RNA 4, collinear with the 3' end of the RNA 3 (Bol, 1999).

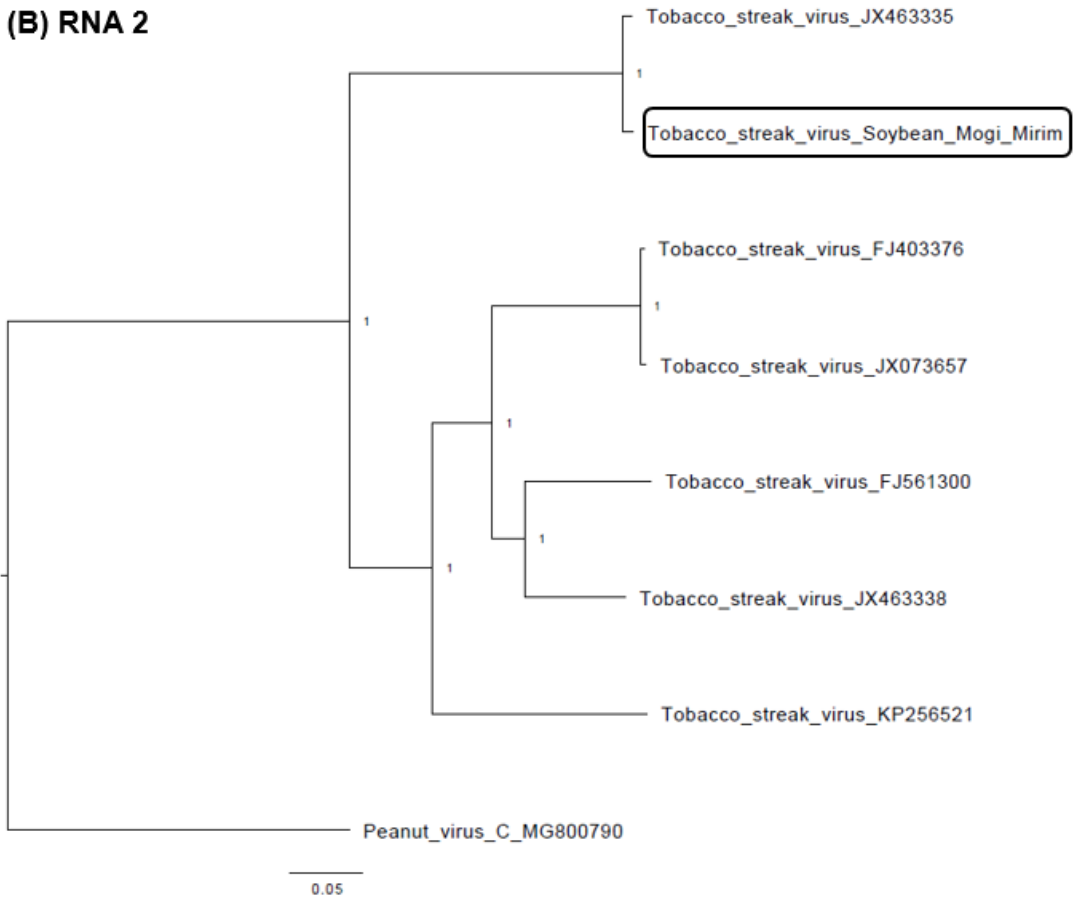
Contigs obtained for the RNA 1, RNA2 and RNA3 of the TSV soybean Mogi Mirim isolate aligned with the GenBank database using BLAST 2.7 showed the highest nucleotide identity (98%) with the Australian TSV isolate from sunflower (Acession numbers: JX463334, JX463335 and JX463336, respectively). The bayesian phylogenetic analysis performed with Mr. Bayers 3.2.2 for the three RNAs confirmed the closest relationship with this isolate indicating a possible common origin. It is interesting to note that according to the CP analysis, TSV Soybean Mogi Mirim share

very high identity with the Brazilian isolate TSV-BR collected from soybean, Accession number AY354406, previously described by (Almeida et al. 2005) (Figure 2).

(A) RNA 1



(B) RNA 2



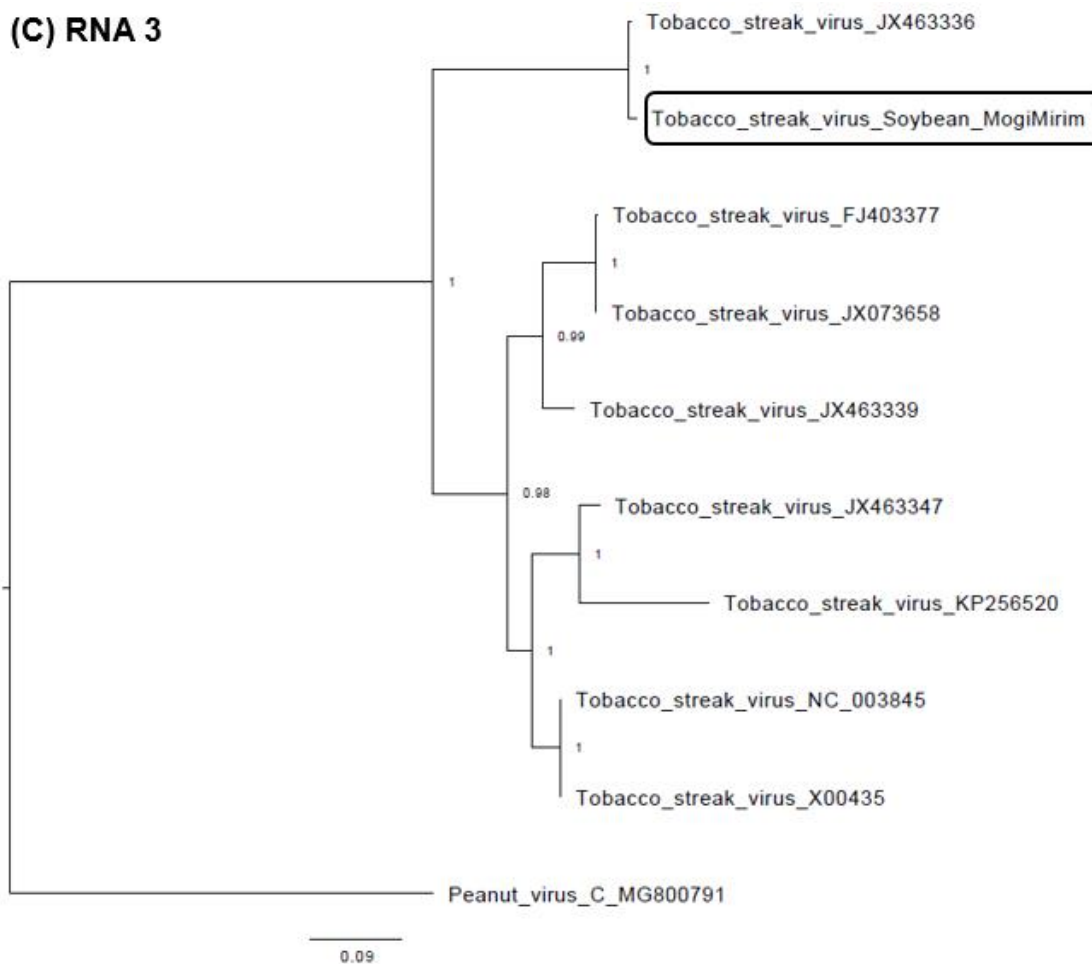
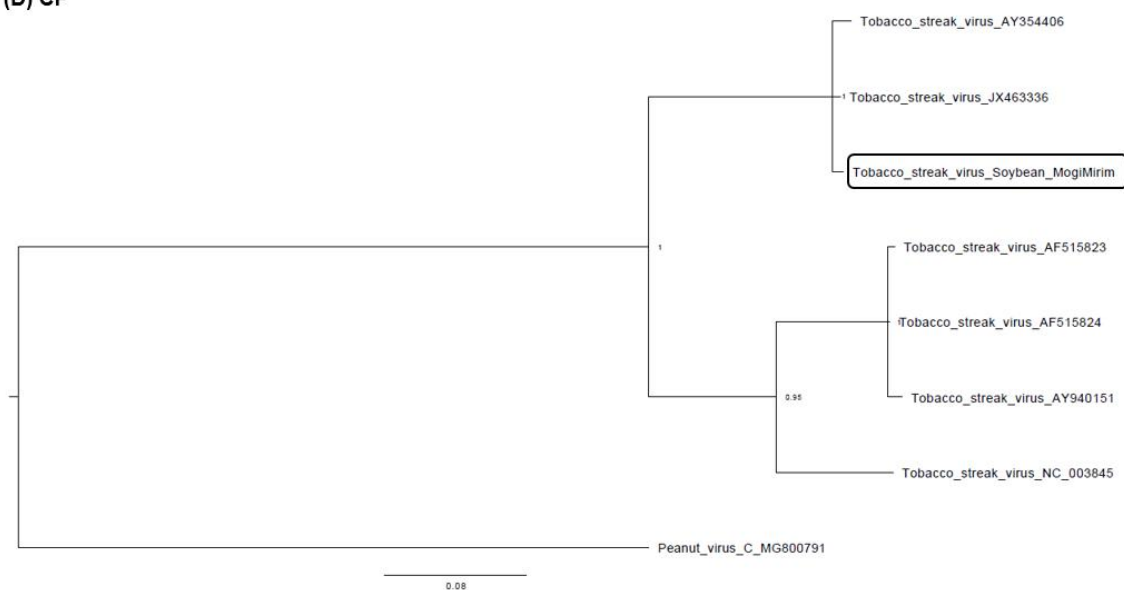
(C) RNA 3**(D) CP**

Figure 2. Phylogenetic trees constructed based on complete sequences of RNA 1 (A), RNA 2 (B), RNA 3 (C) and CP (D) for the Tobacco_streak_virus_Soybean Mogi Mirim isolate and other isolates of *Tobacco streak virus*. The putative novel ilarvirus peanut virus C was used as an out-group.

In the USA there was a sudden revival of TSV in 2016 that might have been influenced by hot and dry weather that is preferred by thrips (Irizarry et al. 2016). In Brazil, the 2018/2019 season was hot and dry, that might have contributed to high populations of thrips and helped to spread GRSV and TSV in the field, as well as for the whitefly *Bemisia tabaci* which might contribute to spread CPMMV.

Diagnosis can be difficult in cases of mix infection as we observed in this study. Therefore, the application of next generation high throughput sequencing technology is a powerful and sensitive tool to detect unsuspected or even unknown viruses (Kreuze et al. 2009; Dutta et al. 2015; Adams et al. 2018; De Marchi et al. 2018).

Study of virus diseases that may affect soybean is important to keep up the soybean yield and further studies are necessary to evaluate the impacts of the TSV in soybeans in Brazil, once that there is not much knowledge about its impact on soybean productivity. This is the first complete genome determined of a Brazilian TSV soybean isolate.

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CAPÍTULO 3 - Uma ameaça oculta à soja: o carlavírus transmitido por mosca-branca, da necrose à ausência de sintomas

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Tipo de artigo: Notícia Técnica

Revista Cultivar Grandes Culturas

A cultura da soja é de grande importância a nível mundial para produção de óleo e como fonte de proteína. O Brasil é o segundo maior produtor de soja no mundo, ficando atrás apenas dos Estados Unidos. No Brasil, a área plantada de soja alcançou 35,1 milhões de hectares, com produção de 119,23 milhões de toneladas na safra 2017/2018.

Com esse aumento do cultivo, várias pragas e doenças se destacaram como agentes preocupantes na produção de soja, incluindo a mosca-branca *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). *Bemisia tabaci* é uma das espécies mais invasivas e disseminadas a nível global, causando grandes prejuízos para diversas espécies vegetais (Foto 1). Por sua polifagia, ou seja, por colonizar várias espécies de plantas, pode estar presente durante o ano todo nos campos brasileiros.



Figura 1. *Bemisia tabaci* MEAM1.

Composta por um complexo de até 43 espécies crípticas morfológicamente indistinguíveis, *B. tabaci* pode ser diferenciada apenas pelo uso da tecnologia molecular, baseando-se em uma parte do genoma mitocondrial, denominado de “mitochondrial cytochrome oxidase I” (mtCOI). Dentre as espécies crípticas, as que ganham maior destaque são as denominadas Middle East-Asia Minor 1 (MEAM1 ou biótipo B) e Mediterranean (MED ou biótipo Q).

No Brasil, a espécie MEAM1 (biótipo B) foi relatada pela primeira vez no início da década de 90, no estado de São Paulo, associada a plantas ornamentais. A espécie MED, por sua vez, foi relatada em 2014 no estado do Rio Grande do Sul em pimentão e, posteriormente, nos estados de São Paulo e Paraná associada a plantas ornamentais. A espécie MEAM1 é a predominante no país inteiro, enquanto que a espécie MED restrita a algumas áreas e culturas nos estados do Rio Grande do Sul, São Paulo, Paraná, Santa Catarina, Minas Gerais e Espírito Santo.



Figura 2. Mapa de distribuição de *Bemisia tabaci* no Brasil.

(Fonte: <https://www.fca.unesp.br/#!/pesquisa/gpmb/mapas/>)

Além da preocupação com a disseminação do inseto, é importante lembrar que a mosca-branca não causa apenas danos diretos nas culturas pela sucção de seiva do floema. Na verdade, o grande destaque dessa praga está na capacidade de transmissão de diversos vírus em culturas variadas, especialmente nas solanáceas e fabáceas. Esse vetor é capaz de transmitir vírus dos gêneros *Begomovirus*, *Carlavirus*, *Crinivirus*, *Ipomovirus* e *Torradovirus*.

Dos vírus transmitidos por mosca-branca destaca-se no feijoeiro o *Bean golden mosaic virus* – BGMV, *Begomovirus*, causador do mosaico dourado do feijoeiro, podendo causar perdas de 100% nas lavouras de feijão. Os sintomas ocasionados pelo BGMV no feijoeiro incluem um forte amarelecimento e redução do crescimento

da planta. Outro vírus que vem merecendo destaque nestes últimos anos, é o *Cowpea mild mottle vírus* (CPMMV), também transmitido por mosca-branca. O CPMMV pertence ao gênero *Carlavirus*, Família *Betaflexiviridae*. O CPMMV foi descrito pela primeira vez no país no final da década de 70, sendo denominado causador da doença da mancha angular do feijoeiro Jalo.

O CPMMV também infecta a soja. Em soja, essa doença foi relatada no Brasil apenas na safra de 2000/01 na região centro-oeste, quando produtores do estado de Goiás observaram plantas com sintomas de nanismo, queima do broto e necrose da haste e até mesmo morte das plantas (Prancha 1 A). Nas safras seguintes a doença foi constatada em diversas regiões produtoras do país como: Tocantins, Pernambuco, Maranhão, Bahia, Distrito Federal, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, São Paulo e Paraná.

Através do melhoramento da soja, a doença da necrose da haste causada pelo CPMMV teve sua importância reduzida, uma vez que os genótipos que respondiam com a necrose sistêmica foram substituídos por outros que não exibiam esse sintoma. Nos últimos anos, porém, sintomas diversificados, característicos de vírus em cultivares de soja começaram a surgir nas principais regiões produtoras. Após análises moleculares, foi detectado o CPMMV em diversas regiões do Brasil, ocasionando sintomas muito diferentes daqueles relatados na literatura no passado.

O Grupo de Pesquisa em Mosca-Branca (GPMB) da Faculdade de Ciências Agronômicas – UNESP, campus de Botucatu em conjunto com a Fundação MT, Instituto Phytus, Universidade Estadual da Bahia e Corteva Agrisciences, têm trabalhado com o CPMMV em soja e sinaliza: o CPMMV pode provocar perdas significativas para a cultura da soja. Neste estudo diversos genótipos comerciais e bastante utilizados nos estados de SP, MT, GO e BA foram avaliados quanto a resposta à infecção com o CPMMV (Prancha 1) e geraram informações importantes para a identificação e entendimento dessa ameaça no campo.

Pôde-se verificar sintomas de mosqueado, clorose, redução do crescimento da planta, redução do número de vagens, redução da massa de grãos, sintomas esses que vão influenciar diretamente a produtividade. As respostas das cultivares não seguiram um padrão definido, e algumas cultivares de soja não mostraram sintomas claros da infecção viral, ou seja, foram assintomáticas. Também foi verificado que

plantas podem mostrar os sintomas de clorose e mosaico nas fases vegetativas e início da reprodutiva, e os sintomas desaparecerem na fase reprodutiva podendo facilmente serem confundidos com fitotoxicidade de produtos aplicados. Além disso, as condições ambientais, estirpes de vírus e infecções mistas com outros vírus implicam em variações dos sintomas causados pelo CPMMV, dificultando assim sua correta identificação.

É interessante ressaltar que a ausência de sintomas visíveis do CPMMV nas folhas de soja, não garante ausência de perdas de produção. Estudos realizados com inoculação do vírus em condições de campo, demonstraram perdas de até 11 sacas por hectare em uma das cultivares, na qual não havia sintomas visíveis da doença, demonstrando que essa pode ser uma doença “silenciosa”.

Essas informações remetem a reflexão de que o produtor pode enfrentar dificuldade de identificar a presença da doença e entender possíveis perdas, uma vez que a “necrose da haste” observada nos estudos com cultivares da época, hoje não é o principal sintoma apresentado nas cultivares atuais.

O aumento da ocorrência do CPMMV no campo está possivelmente relacionado com aumento populacional de mosca-branca, devido às grandes áreas de sucessão das safras de soja e feijão, e vice-versa, em diferentes regiões do país. Durante o ciclo da cultura, veranicos podem, também, favorecer a explosão populacional da praga. Além disso, ambas as culturas são excelentes hospedeiras do inseto, além de ser hospedeiras do vírus. Dessa forma, as populações de mosca-branca e do vírus podem ser mantidas no campo durante todo o ano em soja e feijão.

A transmissão do CPMMV pela mosca-branca é do tipo não-persistente, ou seja, curtos períodos de alimentação do inseto em uma planta infectada já possibilitam a aquisição do vírus, bem como a inoculação em plantas saudáveis. Portanto, tudo pode ocorrer muito rápido no campo.

Além da soja e do feijão, o CPMMV infecta o amendoim (*Arachis hypogea*) e diversas plantas daninhas, dentre elas: guanxuma (*Sida* sp.), *Macropitilium* spp., fedegoso (*Senna* spp.), carrapicho beijo-de-boi (*Desmodium glabrum*), feijãozinho-bravo (*Rhynchosia mínima*), maravilha (*Mirabilis jalapa*), mussambe (*Cleome affinis*), e erva-palha (*Blainville arhomboidea*) que podem servir de reservatório para o vírus

no campo. Além destas espécies, o CPMMV infecta, experimentalmente, outros hospedeiros, como: quinoa (*Chenopodium quinoa*), *C. amaranthicolor*, fumo (*Nicotiniana* spp.), chícharo (*Lathyrus sativum*), ervilha (*Pisum sativum*) e tremçoço (*Lupinus albus*). Esse cenário de abundância de hospedeiros para o vírus e também para o inseto remete à importância do manejo adequado da lavoura levando em consideração as demais disciplinas além de entomologia.

Além da transmissão pela mosca-branca, o CPMMV também pode ser transmitido por sementes de soja, como já relatado em Gana e na Índia. Estudos realizados pelo grupo demonstraram que para pelo menos em um cultivar avaliado foi verificada transmissão via semente de soja do CPMMV o que agrava a epidemiologia, já que a infecção primária da lavoura pode ser via semente. O percentual foi baixo mas merece esforços para melhor entendimento, e devido a isso novos estudos incluindo um número maior de cultivares e novos parâmetros fisiológicos envolvidos estão em fase de execução.

Por fim, esse conjunto de componentes - inseto vetor, vírus, hospedeiros suscetíveis à doença, ambiente climático favorável para a presença do inseto e dos hospedeiros – compõe um patossistema que requer uma abordagem interdisciplinar para que propostas de manejo sejam efetivas. O controle de sanidade das sementes é um critério fundamental para evitar a infecção primária no início do ciclo aliado é claro a cultivares mais tolerantes. As medidas adotadas no cultivo já estabelecido partem do manejo adequado de plantas daninhas para reduzir a fonte de inóculo do vírus, bem como a abundância de hospedeiros alternativos que promovam um aumento exponencial do vetor. Além disso, o controle do vetor se torna fundamental para redução da disseminação da doença.

Embora a detecção do patógeno ainda seja possível somente com emprego de ferramentas moleculares, o grupo está desenhando um mapeamento de ocorrência da doença em diferentes regiões e ofertar informações sobre histórico de inóculo.

O manejo desse patossistema envolve integração de conhecimento e de áreas de atuação e esse novo desafio para a sojicultura pode promover um entendimento mais conectado de conhecimento e profissionais, desde a academia até a extensão, para contribuirmos de maneira efetiva com nossos produtores.



Prancha 1. Sintomas observados em cultivares de soja inoculadas com *Cowpea mild mottle virus* (CPMMV). **A.** BRS 132. **B.** MM 6410 IPRO. **C.** TMG 7062 IPRO. **D.** cv. não identificada coletada em Mori Mirim. **E.** M 8808 IPRO. **F.** MM 8349 IPRO. **G.** M 7739 IPRO. **H.** M 9144 RR **I.** M 9144 RR. **J.** M 9144 RR. **K.** M 8372 IPRO. **L.** BMX PORTÊNCIA RR.

CONSIDERAÇÕES FINAIS

O CPMMV é capaz de reduzir a produtividade da soja.

Os sintomas foliares do CPMMV em soja são variáveis de acordo com a cultivar, podendo ser de clorose, bolhas, enrugamento ou até mesmo assintomáticos.

Os danos causados pelo CPMMV devem ser observados pela avaliação dos aspectos agronômicos da cultura e não somente através dos sintomas foliares.

O TSV encontrado infectando soja no Brasil é filogeneticamente próximo ao TSV da Austrália.

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