



**UNIVERSIDADE FEDERAL RURAL DE  
PERNAMBUCO**

Pró-Reitoria de Pesquisa e Pós-Graduação



**PROGRAMA DE PÓS-GRADUAÇÃO EM  
FITOPATOLOGIA**

**Dissertação de Mestrado**

**Viroma de plantas de amendoim (*Arachis hypogaea*)  
provenientes do Estado de São Paulo**

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**Recife - PE**

**2016**

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**VIROMA DE PLANTAS DE AMENDOIM (*Arachis hypogaea*) PROVENIENTES  
DO ESTADO DE SÃO PAULO**

Dissertação apresentada ao programa de Pós-Graduação em Fitopatologia da Universidade Federal Rural de Pernambuco, como parte dos requisitos para a obtenção do título de Mestre em Fitopatologia.

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**RECIFE-PE  
FEVEREIRO-2016**

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A Deus, por me amparar nos momentos difíceis, pelo fortalecimento interior para superar todas as dificuldades e me suprir em todas as minhas necessidades.

## **AGRADEÇO**

Aos meus pais Eliecê Almeida dos Reis e Lucival Nunes dos Reis pelo amor e apoio incondicional.

## **DEDICO**

À minha sobrinha-irmã Isís Vitória por ser uma das minhas principais motivações nessa vida, aos meus irmãos Luan, Aline e Ticiane, e aos meus avós Deusarina Goes Almeida e Ubiratan Nascimento Almeida (*In memoriam*).

## **OFEREÇO**

## **AGRADECIMENTOS**

Agradeço primeiramente a Deus, por ter me sustentando e me dado forças para vencer mais essa etapa da minha vida;

À Universidade Federal Rural de Pernambuco (UFRPE), pela oportunidade de realizar o Curso de Mestrado, à Coordenação de Aperfeiçoamento de Nível Superior (CAPES), à Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE), pelo apoio concedido e à Universidade de Brasília (UnB), por oferecer o suporte e espaço necessários para a execução do trabalho;

Aos professores de Fitopatologia da UFRPE, pela contribuição na minha formação;

À professora Rita de Cássia Pereira Carvalho, por toda orientação, paciência, amizade, incentivos, ensinamentos e exemplo de profissional e pessoa. Ao professor Gilvan Pio Ribeiro e à Dra. Genira Pereira de Andrade, pela orientação, pelos ensinamentos e atenção. Ao professor Fernando Lucas Mello, pela paciência, atenção e ajuda no trabalho;

Aos familiares que me apoiaram e incentivaram nessa jornada, em especial a minha tia Irecê, tio Wilson e meu primo Leandro Amaro, pelo apoio que me deram e pela torcida para que tudo desse certo;

Aos meus amigos Rafael, Paulo, Gisele, Fernanda, Kamille, Ghaby e Edson Neto, pelas palavras de incentivo e força que me deram durante esses dois anos. Aos amigos da UFRPE Emanuel, Leticia, Moara, Claudeana e Michelle, pela força, amizade e carinho;

A “dona” Nazaré, pela atenção e pelo carinho. Aos amigos da UnB Geisianny, Catharine, Pimentel, Cristina, Felipe, Pedro, Bruna, Nancy, Cecília e Rayane e a todos os colegas que foram tão gentis comigo no decorrer desse tempo de desenvolvimento dos trabalhos;

Às meninas do Laboratório de Virologia Vegetal, Caroline, Flávia e a Josiane, pela amizade, companheirismo, carinho, atenção e por sempre estarem dispostas a me ajudar. Aos funcionários da Estação Experimental de Biologia da UnB, Sr. Fábio, “dona” Olinda e Evandro, pela amizade e pela contribuição essencial na condução dos experimentos. E a todos que, de alguma forma, contribuíram com o trabalho.

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## RESUMO GERAL

O amendoim (*Arachis hypogaea* L.) é uma importante fonte de proteína e óleo, sendo considerada uma das plantas mais cultivadas no Brasil e no mundo, com grande diversidade de formas de consumo de seus produtos. É a quarta oleaginosa com maior produção mundial, cultivada em mais de 30 países, em que a China lidera com 40,8%, seguida pela Índia com 14% e Nigéria com 7,5%. O Brasil ocupa a 17ª posição, concentrada nas regiões Sudeste e Sul, sendo o Estado de São Paulo responsável por 91,4% da produção nacional. Dentre os fatores que limitam o desenvolvimento dessa cultura, encontram-se diversos vírus, os quais ocasionam a redução da produtividade e valor do produto para comercialização. Até o momento, já foram registradas, infectando naturalmente o amendoim, 31 espécies virais, classificadas nos seguintes gêneros e respectivas famílias: *Begomovirus* (*Geminiviridae*), *Bromovirus* (*Bromoviridae*), *Carlavirus* (*Betaflexiviridae*), *Cucumovirus* (*Bromoviridae*), *Irlavirus* (*Bromoviridae*), *Luteovirus* (*Luteoviridae*), *Pecluvirus* (*Virgaviridae*), *Potexvirus* (*Alphafleviridae*), *Potyvirus* (*Potyviridae*), *Rhabdovirus* (*Rhabdoviridae*), *Soymovirus* (*Caulimoviridae*), *Tospovirus* (*Bunyaviridae*), *Tymovirus* (*Tymoviridae*) e *Umbravirus* (*Tombusviridae*). Destes, o gênero *Potyvirus* é o que agrega maior número de espécies. O advento da metagenômica, aliada às tecnologias de alto desempenho (*Next Generation Sequencing* - NGS) vêm propiciando a exploração do universo de microrganismos em várias áreas das ciências, inclusive na Virologia Vegetal. Estas técnicas, juntamente com a bioinformática, atuam como excelentes ferramentas para detecção e caracterização de novos vírus e de todos os genomas virais presentes em determinadas amostras, sejam de diferentes espécies ou estirpes. O objetivo desse trabalho foi realizar estudo de viroma de plantas de amendoim exibindo sintomas típicos de viroses, obtidas de áreas produtoras de 10 municípios do Estado de São Paulo por NGS. Os isolados virais foram mantidos por sucessivas passagens para plantas de amendoim por meio de enxertia, em casa de vegetação da Universidade de Brasília (UnB). Para a realização do NGS, as amostras foram semipurificadas objetivando um enriquecimento de partículas virais seguido de extração de RNA total e o sequenciamento, realizado pela Plataforma Illumina Miseq. A análise metagenômica permitiu a detecção do genoma completo do *Peanut mottle virus* - PeMoV (*Potyvirus*) e do *Groundnut ringspot virus* - GRSV (*Tospovirus*). Análises biológicas (plantas indicadoras), sorológicas (Dot-ELISA) e moleculares (RT-PCR) foram realizadas com intuito de caracterizar alguns isolados de GRSV de amendoim cuja sequência foi identificada por metagenômica. Análises moleculares do gene que



codifica a proteína N, amplamente utilizado em trabalhos de taxonomia no gênero *Tospovirus*, foram também realizadas com os isolados selecionados A1, N1 e O1. Estes três isolados induziram reação sintomatológica em pelo menos uma das espécies indicadoras (*Datura stramonium* L.) utilizadas nesse trabalho e amplamente citada na literatura. Resultados positivos para estes isolados foram também confirmados por sorologia, evidenciando a presença de GRSV em municípios produtores de amendoim no Estado de São Paulo. Análises filogenéticas para os segmentos L, M e S de tospovírus revelaram que o isolado de GRSV de amendoim estudado neste trabalho agrupou-se com sequências de um isolado de GRSV de melancia (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) e um isolado recombinante de GRSV e *Tomato chlorotic spot virus* - TCSV, obtido de tomate (*Solanum lycopersicum* L.). Entretanto, com a análise filogenética feita com a sequência da proteína N, ficou demonstrado que o presente isolado de amendoim se agrupou com sequências de isolados de GRSV previamente estudado no Brasil. Após análises filogenéticas para as proteínas de todos os segmentos de GRSV, acredita-se que as espécies de GRSV e TCSV compartilham o mesmo segmento M. Comparação das sequências da capa proteica de PeMoV, revelaram que o isolado brasileiro de amendoim relatado neste trabalho está mais relacionado filogeneticamente com um isolado obtido de soja (*Glycine max* L.), encontrado na Coreia do Sul, apresentando 98% de identidade. Este é o primeiro relato no Brasil e no Mundo das sequências do genoma completo de PeMoV e GRSV em amendoim, por meio do uso de NGS.

**Palavras chaves:** Metagenômica, NGS, GRSV, PeMoV, *Tospovirus*

## GENERAL ABSTRACT

The peanut (*Arachis hypogaea* L.) is an important source of protein and oil, and considered one of the most cultivated plants in Brazil and worldwide, that provide products consumed in different ways. It is the fourth world largest oleaginous in seed production, cultivated in over 30 countries, where China leads with 40.8%, followed by India with 14% and Nigeria with 7.5%. Brazil occupies the 17<sup>th</sup> position, concentrated in the Southeast and South regions, and the State of São Paulo accounts for 91.4% of national production. Among the factors that limit the development of this crop, there are several viruses, which cause reduction on productivity and the value of the product for marketing. Up to now, 31 viral species, classified in the following genera and respective families have been recorded naturally infecting peanut: *Begomovirus* (*Geminiviridae*), *Bromovirus* (*Bromoviridae*), *Carlavirus* (*Betaflexiviridae*), *Cucumovirus* (*Bromoviridae*), *Irlavirus* (*Bromoviridae*), *Luteovirus* (*Luteoviridae*), *Pecluvirus* (*Virgaviridae*), *Potexvirus* (*Alphafleviridae*), *Potyvirus* (*Potyviridae*), *Rhabdovirus* (*Rhabdoviridae*), *Soymovirus* (*Caulimoviridae*), *Tospovirus* (*Bunyaviridae*), *Tymovirus* (*Tymoviridae*) e *Umbravirus* (*Tombusviridae*). Of these genera, *Potyvirus* has more species. The advent of metagenomics, combined with high-performance technologies (Next Generation Sequencing - NGS) provides the exploration of micro-universe in various areas of science, including the Plant Virology. These techniques, together with bioinformatics, act as excellent tools for detection and characterization of novel viruses, as well as, all the viral genomes of different species or strains present in certain samples. The objective of this work was to study the virome of peanut plants exhibiting typical virus symptoms, obtained from producing areas of 10 counties of the State of São Paulo by NGS. The viral isolates were maintained by successive passages to peanut plants by grafting, under greenhouse conditions at the University of Brasilia (UnB). To perform the NGN, samples were semipurified aiming enrichment of virus particles, followed by total RNA extraction and sequencing carried out by Illumina platform. The metagenomic analysis permitted the detection the complete genome of *Peanut mottle virus virus* - PeMoV (*Potyvirus*) and *Groundnut ringspot virus* - GRSV (*Tospovirus*). Biological (indicator plants), serological (Dot-ELISA) and molecular (RT-PCR) tests were performed in order to characterize some GRSV isolates from peanut, which sequences were identified by metagenomic. Molecular analysis of the gene encoding the N protein, used for species taxonomy within

*Tospovirus* genus, was also carried out with the selected isolates A1, N1 and O1. These three isolates induced symptomatic response in at least one of the indicator species (*Datura stramonium* L.) used in this work and widely cited in the literature. Positive results for these isolates were also confirmed by serology, indicating the presence of GRSV in peanut producing counties in the State of São Paulo. Phylogenetic analyzes of the segments L, M and S of tospoviruses revealed that the peanut GRSV isolates studied in this work grouped with sequences of GRSV isolate from watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) and a recombinant isolate of GRSV and *Tomato chlorotic spot virus* - TCSV obtained from tomato (*Solanum lycopersicum* L.). However, the phylogenetic analysis using the N protein sequence, demonstrated that the present peanut isolate grouped with sequences of GRSV isolates previously studied in Brazil. After phylogenetic analysis for proteins of all GRSV segments, it is believed that the species GRSV and TCSV share the same M segment. The comparison of PeMoV coat protein sequences revealed that the Brazilian peanut isolate reported in this work is more phylogenetically related with the isolate from South Korea, obtained from soybean (*Glycine max* L.) with 98% identity. This is the first report in Brazil and in the world of the complete genome sequences of PeMoV and GRSV in peanuts, by using NGS.

**Keywords:** Metagenomics, NGS, GRSV, PeMoV, *Tospovirus*



## **CAPÍTULO I**



### **Introdução Geral**

## VIROMA DE PLANTAS DE AMENDOIM (*Arachis hypogaea*) PROVENIENTES DO ESTADO DE SÃO PAULO

### INTRODUÇÃO GERAL

#### 1. Aspectos culturais e econômicos do amendoim

O amendoim (*Arachis hypogaea* L.) é uma dicotiledônea herbácea anual de ciclo indeterminado pertencente à família Fabaceae, subfamília Papilionoidea (GREGORY et al., 1980; SANTOS, 2010; KRISHNA et al., 2015). Originário da América do Sul, esta leguminosa tem como centro de origem o sudeste da Bolívia e noroeste da Argentina, com registro de *A. ipaensis* Krapov. & W. C. Greg. E *A. duranensis* Krapov. & W.C. Greg. como possíveis ancestrais (FÁVERO et al., 2006).

O amendoim apresenta uma estrutura de frutificação dotada de geotropismo positivo, denominada de ginóforo, a qual é responsável por transportar a vagem na extremidade, fazendo com que o fruto amadureça sob a superfície do solo. Os genótipos do tipo agrícola Virgínia, pertencente à subespécie *hypogaea*, possuem ramificações vegetativas alternadas nos ramos primários. Por outro lado, os genótipos pertencentes aos tipos agrícola Valência e Spanish, da subespécie *fastigata*, expõem flores sobre o eixo central, apresentando ramificações reprodutivas e vegetativas desorganizadas ao longo dos ramos primários (VALLS, 2013).

O sistema reprodutivo do amendoim é formado por flores hermafroditas e cleistogâmicas permitindo a ocorrência de autofecundação com baixa taxa de polinização cruzada, menos que 1% (NIGAM et al., 1990). Os frutos variam quanto à forma, tamanho e número de sementes em cada vagem e sua maturação ocorre em função do ciclo mais ou menos longo dos genótipos, sendo as cultivares dos tipos Spanish e Valência mais precoces, enquanto as do tipo Virgínia apresentam ciclo mais tardio (SANTOS; GODOY; FÁVERO, 2005). As sementes do amendoim apresentam um sabor agradável com colorações diversificadas, que vão do bege ao vermelho, utilizadas para diversos fins, sendo caracterizadas pelo seu alto teor proteico. As folhas e caule são utilizados como forragens (SANTOS; MELO FILHO; GOMES, 2007), enquanto o bagaço é aproveitado na forma de torta para alimentação animal (NAKAGAWA; ROSOLEM, 2011).

A semente de amendoim é composta por cerca de 40 a 50% de óleo, 27 a 33% de proteína, assim como vitaminas e minerais essenciais (NAGAWA; ROSOLEM, 2011; FERREIRA, 2014). Devido à tendência mundial da utilização de recursos renováveis como matriz energética, a cultura do amendoim, pode ser inserida em programas de produção de biocombustíveis, como por exemplo, a exploração de óleos vegetais como matéria-prima para produção de biodiesel, pelo fato de apresentar altos índices de ácidos graxos (MELO FILHO; SANTOS, 2010).

A importância do amendoim no mercado alimentício reside na versatilidade de aplicações, a qual possibilita as várias formas de consumo sendo o grão muito apreciado na alimentação humana, consumido *in natura* torrado, cozido ou frito, salgado ou sem sal, e em produtos industrializados na forma de doces, paçocas, chocolates, biscoitos, pastas, maionese, margarina, gordura hidrogenada e cremes (PATTEE, 2005). Além disso, o amendoim pode ser usado nas indústrias de cosméticos e farmacêuticos (CAMPESTRE, 2010).

Essa oleaginosa, apreciada em todo o mundo é a quarta mais produzida e cultivada em mais de 30 países (MARTINS, 2013). É uma cultura que se sobressai em área plantada 24.709.458 hectares (ha) com produção total de 41.185.933 toneladas (t) e uma produtividade total de 1.667 kg/ha. A China lidera a produção mundial, com 40,8%, seguida pela Índia com 14% e Nigéria com 7,5%. Na China 95% do que é produzido destina-se ao consumo interno e o restante segue para a exportação (CONAB, 2015). Os maiores importadores mundiais de amendoim são Holanda, Reino Unido, Canadá, Japão e Singapura. Os principais exportadores são a China, os Estados Unidos e Argentina, principalmente para o Japão e Europa, devido à alta qualidade dos grãos (SANTOS et al., 2012; FAO, 2014).

O Brasil ocupa a 17ª posição na produção mundial de amendoim com uma área plantada de 110.336 ha, produção total de 334.224 t e uma produtividade total de 3.028 kg/ha. A sua produção está concentrada nas regiões Sudeste e Sul, principalmente nos Estados de São Paulo, Minas Gerais e Paraná. Em São Paulo, o maior produtor de amendoim do país, cerca de 90,0% da produção destina-se ao mercado europeu. Atualmente, o Brasil exporta 40% do amendoim produzido e os demais 60% são destinados ao consumo interno. Em Minas Gerais, a região conhecida como triângulo mineiro responde por 80% da área de cultivo e por 93% do volume de produção do estado, incluindo lavouras com sementes de alta qualidade (IAC 505), que são plantadas normalmente em novembro e dezembro, com colheita entre março e maio (FAO, 2014; CONAB, 2015).

Dentre os fatores que limitam a produção dessa cultura em várias partes do mundo estão às doenças que podem se apresentar tanto na fase de plantio, quanto durante o desenvolvimento da planta. Os patógenos que afetam o amendoim incluem fungos, vírus, nematoides, bactérias e um fitoplasma (PIO-RIBEIRO et al., 2013).

As viroses vêm sendo relatadas mais frequentemente nessa cultura ocasionando problemas importantes como a redução do seu desenvolvimento e conseqüentemente a produtividade (THIESSEN; WOODWARD, 2012). Em nível mundial, já foram registradas infecções naturais de 31 espécies de vírus (Tabela 1), classificadas nos seguintes gêneros e respectivas famílias: O gênero *Potyvirus* (*Potyviridae*) é o que agrega maior número de espécies, seguido por *Tospovirus* (*Bunyaviridae*), *Cucumovirus* (*Bromoviridae*), *Pecluvirus* (*Virgaviridae*), *Soymovirus* (*Caulimoviridae*), *Umbravirus* (*Tombusviridae*), *Begomovirus* (*Geminiviridae*), *Bromovirus* (*Bromoviridae*), *Rhabdovirus* (*Rhabdoviridae*), *Ilarvirus* (*Bromoviridae*), *Potexvirus* (*Alphafleviridae*), *Tymovirus* (*Tymoviridae*), *Carlavirus* (*Betaflexiviridae*) e *Luteovirus* (*Luteoviridae*) (SREENIVASULU; REDDY, 2008; PIO-RIBEIRO et al., 2013; ICTV, 2014).

No Brasil, já foram relatadas em amendoim seis espécies virais pertencentes aos seguintes gêneros: *Irlavirus* - *Tobacco streak virus* (TSV); *Potyvirus* - *Cowpea aphid-borne mosaic virus* (CABMV), *Peanut mottle virus* (PeMoV) e *Bean common mosaic virus*, estirpe *Peanut stripe* (BCMV-PSt) e *Tospovirus* - *Tomato spotted wilt virus* (TSWV) e *Groundnut ringspot virus* (GRSV) (COSTA, 1941; COSTA; CARVALHO, 1961; PIO-RIBEIRO et al., 1996; COSTA; KITAJIMA, 1974; ANDRADE et al. 1997a; ANDRADE et al. 1997b; PIO-RIBEIRO et al., 2000; ANDRADE et al., 2003).

## **2. Principais gêneros de vírus com espécies que afetam a cultura do amendoim**

### **2.1. Gênero *Potyvirus***

Em número de espécies, o gênero *Potyvirus* é o maior da família *Potyviridae*, que por sua vez ocupa a segunda posição entre os vírus de plantas, incluindo agentes de doenças em várias culturas anuais, perenes de clima tropical e temperado, como hortaliças e fruteiras (NETO-FRISCHE; BORÉM, 2012; KING et al., 2012). Atualmente, a família *Potyviridae* é constituída por oito gêneros: *Brambyvirus*, *Bymovirus*, *Ipomovirus*, *Macluravirus*, *Poacevirus*, *Potyvirus*, *Rymovirus* e *Tritimovirus*, abrigando 190 espécies, cuja classificação é baseada no agente vetor e na organização do genoma (ICTV, 2014).



Os membros dessa família formam corpos de inclusão no citoplasma das células infectadas. Devido ao seu formato essas inclusões cilíndricas podem ser chamadas de estruturas tipo “cata-vento”, quando observadas em cortes ultrafinos. Os sintomas característicos presentes nas plantas infectadas por espécies da família *Potyviridae* são mosaico, mosqueado, clorose, necrose e deformação de frutos e folhas (SHUKLA et al., 1994; NETO-FRISCHE; BORÉM, 2012).

A transmissão de espécies do gênero *Potyvirus* é realizada por afídeos em uma relação caracterizada como não circulativa e não persistente, mediada pelas proteínas Hc-Pro (Helper component – protein) e CP (Coat protein) ou componente auxiliar e proteína capsidial, respectivamente. As espécies deste gênero possuem partículas alongadas, flexuosas com comprimento entre 680 a 900 nm e de 11 a 13 nm de diâmetro, com genoma constituído por uma única molécula de RNA de fita simples, senso positivo com aproximadamente 10.000 nt. O RNA genômico é envolto por um capsídeo composto por cerca de 2.000 cópias da proteína capsidial. O RNA dos potyvírus é ligado a uma proteína de origem viral VPg (viral protein genome-linked), na extremidade 5' e apresenta uma cauda poliadenilada, também denominada Poli (A) de origem viral na extremidade 3'. A VPg e a CP são os únicos produtos gênicos que participam da constituição da partícula viral (HOLLINGS; BRUNT, 1981; DI PIERO et al., 2006; SOUMYA et al., 2014).

Para classificação de espécies na família *Potyviridae* devem ser seguidos os critérios sequência de aminoácidos da CP menor que 80% de identidade; sequência de nucleotídeo menor que 76% de identidade e diferenças no sítio de clivagem da poliproteína (KING et al., 2012).

Dentre os membros do gênero *Potyvirus* que infectam a cultura do amendoim destaca-se o PeMoV, que está amplamente disseminado nas regiões produtoras. Esta espécie viral foi primeiramente descrita nos Estados Unidos (KUHN, 1965) e possui como hospedeiros naturais a soja (*Glycine max* L.), ervilha (*Pisum sativum* L.), feijão (*Phaseolus vulgaris* L.), cássia (*Cassia occidentalis* L.), *Nicotiana clevelandii* A. Gray e *Chenopodium amaranticolor* Coste e Reyn. (SPIEGEL et al., 2008; BOCK; KUHN, 1975). Este vírus é transmitido principalmente pelas espécies de afídeo *Aphis craccivora*, *A. gossypi*, *Hyperomyzus lactucae*, *Myzus persicae* e *Rhopalosiphum padi* (COSTA; KITAJIMA, 1974; SREENIVASULU; REDDY, 2008; BEIKZADEH; HASSANI-MEHRABAN; PETERS, 2015).

No Brasil, o PeMoV foi referido por Kitajima (1986) como “peanut mosaic” e detectado por Andrade et al. (1996) e Nascimento et al. (2001), infectando amendoim nos estados de São Paulo e Paraíba, respectivamente. Além das detecções em amendoim da espécie *A. hypogaea*, este vírus foi também encontrado em amendoim forrageiro (*A. pintoi* Krapovi.) (ANJOS et al., 1998) e em soja (SOUSA; LIMA; CAMPOS, 1996). Nestes trabalhos a identificação foi realizada por diferentes métodos incluindo testes sorológicos, inoculação em gama de hospedeiros indicadores e microscopia eletrônica.

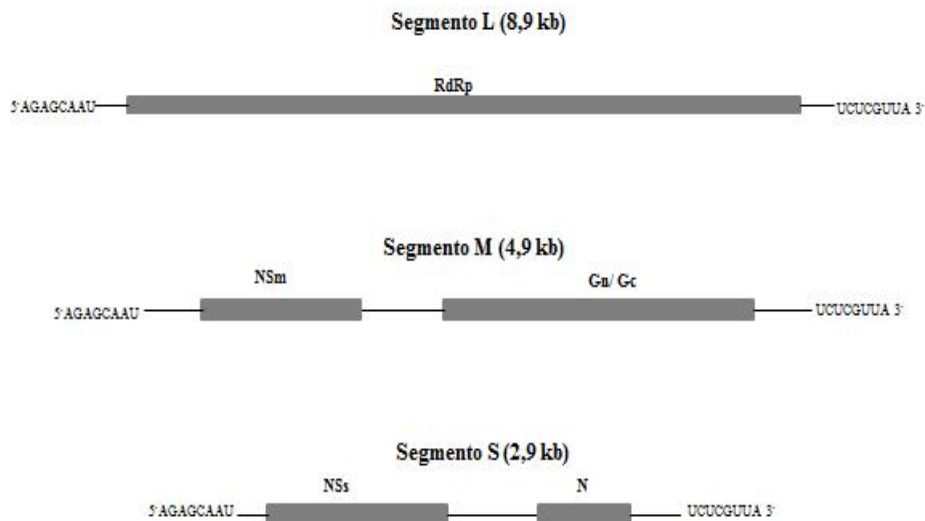
Como principais sintomas provocados pelo PeMoV em amendoim, observam-se manchas irregulares escuras nas folhas, referidas como mosqueado ou mosaico, depressões internervais e enrolamento das margens dos folíolos. Entretanto, relata-se que, apesar desse vírus não causar redução drástica do porte das plantas, tanto o número como o tamanho das vagens podem ser bastante reduzidos. Em campo, há certa dificuldade para reconhecimento desses sintomas porque normalmente são mascarados por outras doenças ocasionadas por diferentes patógenos, sendo adequado e necessário para a identificação da virose, o uso de métodos como microscopia eletrônica, testes sorológicos e moleculares (FERREIRA, 2014; SPIEGEL et al., 2008).

O genoma completo do PeMoV foi sequenciado a primeira vez por Flasinaky; Gonzales; Cassidy (1998) a partir de planta de *Cassia* sp. nos Estados Unidos. Outra sequência completa recente desse vírus depositada no GenBank foi realizada por Lim et al. (2014) de um isolado de soja na Coreia do Sul. Nenhuma das duas sequências se refere a isolado obtido de amendoim no Brasil.

## **2.2. Gênero *Tospovirus***

O gênero *Tospovirus* pertence à família *Bunyaviridae*, a qual é a maior família de arbovírus (*arthropode-borne virus*) de RNA com aproximadamente 350 espécies descritas até o momento (WALTER; BARR, 2011), e está dividida em cinco gêneros: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* que agrupam espécies virais que infectam humanos e animais e *Tospovirus* que infectam plantas (GUU; ZHENG; TAO, 2012; ICTV, 2014). Os vírus desse gênero apresentam partículas esféricas, medindo de 80-120 nm de diâmetro, e apresentam envelope. O genoma é dividido em três segmentos de RNA de fita simples (ssRNA) denominados: S (small) com 2,9 kb, M (medium) com 4,9 kb, ambos com

orientações ambisenso e L (large) 8,9 kb com orientação senso negativo (Figura 1) (SOELLICK et al., 2000; KING et al., 2012).



**Figura 1.** Esquema representativo com os tamanhos aproximados do genoma de membros do gênero *Tospovirus*, mostrando as fases abertas de leitura (Open Reading Frame-ORFs) para as proteínas virais e os oito nucleotídeos complementares que estão presentes nas extremidades dos segmentos (Ilustração adaptada do ViralZone).

O genoma trissegmentado de RNA apresenta cinco fases abertas de leituras (ORF) que codificam seis proteínas virais no total, sendo que quatro delas são estruturais e estão presentes na partícula viral madura como a proteína do nucleocapsídeo (N), RNA polimerase dependente de RNA (RdRp) e as glicoproteínas (Gn/Gc). As proteínas não estruturais são as proteínas responsáveis pelo movimento viral célula-a-célula (NSm) e supressora do silenciamento gênico (NSs). As proteínas NSm e NSs foram caracterizadas como genes de avirulência (Avr) para dois genes de resistência a tospovírus que são o *Tsw* encontrado em pimenta (*Capsicum chinese* Jacq.) e o *Sw-5* encontrado em tomate (*Solanum lycopersicum* L.) (LOPEZ et al., 2011; DE RONDE et al., 2013). A proteína NSm foi confirmada como determinante para o gene *Sw-5* e a NSs para o *Tsw* (HALLWASS, et al., 2014; DE RONDE, et al., 2014).

O segmento L codifica a RdRp, o segmento S codifica a proteína N e a proteína NSs e o segmento M a Gn/Gc e a NSm (Figura 1). Os segmentos S e M apresentam uma região intergênica rica em nucleotídeos adenina e uracila e por consequência desse fato, forma-se nessa região uma estrutura semelhante a um grampo (*hairpin*), estrutura importante no processo da replicação (FAUQUET et al., 2005; SNIPPE et al., 2007).

As espécies do gênero *Tospovirus* são transmitidas por insetos vetores conhecidos popularmente como tripes (ordem: Thysanoptera/família: Thripidae). Já foram descritas aproximadamente 5.500 espécies de tripes distribuídas em todos os continentes, entretanto, apenas 15 espécies, classificadas nos gêneros *Thrips*, *Frankliniella*, *Scirtothrips* e *Ceratothripoides*, foram identificadas como vetores de tospovírus até o momento. Além de *Tospovirus*, os tripes podem transmitir espécies dos gêneros: *Ilarvirus*, *Sobemovirus*, *Carmovirus* e *Machlomovirus* (JONES, 2005; KING et al., 2012).

A transmissão de tospovírus ocorre de maneira circulativa propagativa. Os tripes só adquirem o vírus no estágio larval, através da alimentação em folhas ou outros tecidos de plantas infectadas e os insetos adultos virulíferos continuam transmitindo até o fim do ciclo vital. Contudo, não há transmissão do vírus para prole, ou seja, não se observa transmissão vertical (WIJKAMP et al., 1993; WHITFIELD et al., 2005; MORSE; HODDLE, 2006; KING et al., 2012).

Os tospovírus possuem uma ampla distribuição geográfica. Espécies como TSWV, *Impatiens necrotic spot virus* (INSV) e *Iris yellow spot virus* (IYSV) já foram relatadas nos

cinco continentes (PAPPU; JONES; JAIN, 2009). A espécie GRSV encontra-se distribuída nos continentes americano, europeu e africano (EPPO, 2016).

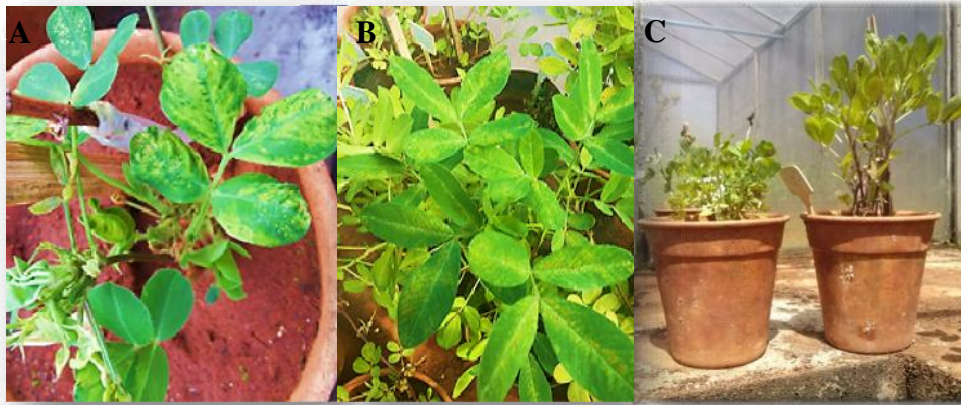
O gênero *Tospovirus* abrange espécies causadoras de doenças em uma ampla gama de plantas cultivadas e silvestres, incluindo várias culturas de grande importância econômica, como é o caso do tomate, pimenta, pimentão (*Capsicum annuum* L.), cebola (*Allium cepa* L.), fumo (*Nicotiana tabacum* L.), abobrinha (*Cucurbita pepo* L.), melancia (*Citrullus lanatus* L.), melão (*Cucumis melo* L.), batata (*Solanum tuberosum* L.), cubiu (*Solanum sessiliflorum* Dunal), amora (*Morus* sp.), amendoim, soja, feijão e plantas ornamentais como cipó enredadeira (*Polygonum convolvulus* L.), lírio-aranha (*Hymenocallis littoralis* L.), copo-de-leite (*Zantedeschia aethiopica* L.), íris e crisântemo (SILVA, et al., 2000; BOARI et al., 2002; OKUDA et al., 2006; PAPPU et al., 2009; ZHOU et al., 2011; CHEN et al., 2012; DE OLIVEIRA et al., 2012; XU et al., 2013; DE JONGHE; MORIO; MAUES, 2013; SPADOTTI et al., 2014; MENG et al., 2015).

Os critérios de classificação taxonômica para a determinação de uma nova espécie no gênero *Tospovirus* baseiam-se em diferenças no espectro de hospedeiros, sintomatologia, transmissão por tripes, propriedades sorológicas e na identidade da sequência da proteína N, que deve ser menor que 90%, quando comparada com as sequências de espécies conhecidas (KING et al., 2012; HASSANI-MEHRABAN et al., 2010).

Com base nesses critérios, um total de 30 espécies de *Tospovirus* já foram identificadas, sendo dez aceitas e vinte e uma consideradas espécies tentativa (ICTV, 2014; MENG et al., 2015), como: TSWV, GRSV, *Tomato chlorotic spot virus* - TCSV (DE ÁVILA et al., 1993), INSV (LAW; SPECK; MOYER, 1991), *Groundnut bud necrosis virus* - GBNV (HEINZE et al., 1995), *Watermelon silver mottle virus* - WSMoV (YEH; CHANG, 1995), *Watermelon bud necrosis virus* - WBNV (JAIN et al., 1998), IYSV (CORTÊZ et al., 1998), *Groundnut yellow spot virus* - GYSV (SATYANARAYANA et al., 1998), *Zucchini lethal chlorosis virus* - ZLCV (BEZERRA et al., 1999), *Chrysanthemum stem necrosis virus* - CSNV (BEZERRA et al., 1999), *Groundnut chlorotic fan-spot virus* - GCFSV (CHU et al., 2001), *Alstrometria necrotic streak virus* - ANSV (HASSANI-MEHRABAN et al., 2010), *Pepper necrotic spot virus* - PNSV (TORRES et al., 2012), *Soybean vein necrosis associated virus* - SVNv (ZHOU et al., 2011), *Hippeastrum chlorotic ring virus* - HCRV (XU et al., 2013.), *Bean necrotic mosaic virus* - BeNMV (DE OLIVEIRA et al., 2011), *Tomato necrotic spot virus* - TNSV (Yin et al., 2014), *Lisianthus necrotic ringspot virus* - LNRV (SHIMOMOTO;

KOBAYASHI; OKUDA, 2014), Calla lily chlorotic spot virus - CCSV (CHEN et al., 2015) e Mulberry vein banding associated virus - MBaV (MENG et al., 2015).

A cultura do amendoim é afetada por TSWV, GRSV, GBNV, GYSV e uma espécie tentativa GCFSV (CHU et al., 2001; PIO-RIBEIRO et al., 2013; ICTV, 2014). Os sintomas de GRSV e TSWV tais como de outros tospovírus que infectam plantas de amendoim podem variar, incluindo desde anéis concêntricos, manchas cloróticas em folíolos, mosaico, deformação foliar, nanismo, descoloração, bastante reduzido o número de vagens produzidas, o tamanho de amêndoas e a produção por planta (Figura 2) (DE BREUIL et al., 2007; PIO-RIBEIRO et al., 2013; CAMELO-GÁRCIA et al., 2014).



**Figura 2.** Ilustrações de sintomas de *Groundnut ringspot virus* - GRSV (gênero *Tospovirus*) em amendoim (*Arachis hypogaea*): a) Mosaico; b) Manchas cloróticas; e c) Nanismo (Almeida-Reis, 2015).

O primeiro relato de um tospovírus foi feito na Austrália em 1915 no tomateiro (BRITTLEBANK, 1919), o qual posteriormente foi denominado de *Tomato spotted wilt virus* (SAMUEL; BALD; PITTMAN, 1930). No Brasil, o primeiro relato de um vírus desse gênero foi em plantações de amendoim efetuadas na Estação Experimental Central do Instituto Agrônomo de Campinas-SP, em 1941, sendo denominada de “mancha anular”. Em estudos posteriores feitos por Costa (1950) esta doença foi associada ao vírus do vira-cabeça (COSTA, 1941).

Dentre as espécies de *Tospovirus* que afetam o amendoim encontra-se GRSV, que foi relatada a primeira vez nesta leguminosa na África do Sul (DE ÁVILA et al., 1993). Na Argentina, o GRSV já foi detectado não só nesta cultura (DE BREUIL et al., 2007) como também em soja (PAPPU et al., 2009). No sul da Flórida, o GRSV foi relatado pela primeira

vez em tomate, e posteriormente em pimenta, tomatillo (*Physalis philadelphica* L.) e berinjela (*Solanum melogena* L.) (WEBSTER et al., 2010; WEBSTER et al., 2011). O GRSV é transmitido principalmente pelas espécies de tripses *Frankliniella schultzei* e *Frankliniella occidentalis* (NAGATA et al., 2004).

A gama de hospedeiros já relatada para GRSV é bem menor do que para TSWV, que infecta uma grande diversidade de espécies de plantas dicotiledôneas e monocotiledôneas. No Brasil, o GRSV já foi relatado em culturas como tomate, fumo (SILVA, et al., 2000), pimenta, pimentão, cubiu, coentro (*Coriandrum sativum* L.), e em pepino (SPADOTTI et al., 2014). Em amendoim o GRSV foi detectado sorologicamente em *Arachis* spp. no Distrito Federal (ANDRADE et al. 1997a), em *A. hypogaea* na Paraíba (ANDRADE et al. 1997b) e em *Arachis* sp. em Pernambuco (ANDRADE et al., 2003). Recentemente houve outro relato dessa espécie viral infectando plantas de *A. hypogaea* em Itápolis no Estado de São Paulo (CAMELO-GARCÍA et al., 2014).

**Tabela 1.** Distribuição e características de vírus já relatados infectando naturalmente a cultura do amendoim (*Arachis hypogaea*) no Brasil e no Mundo (Adaptado de SREENIVASULU et al., 2008)

<b>Espécies de vírus (Acrônimos e Sinônimos)</b>	<b>Gênero/Família</b>	<b>Distribuição</b>	<b>Transmissão</b>	<b>Referências</b>
<i>Bean golden yellow mosaic virus</i> (BGYMV) ou [Peanut yellow mosaic virus]	<i>Begomovirus</i> (Geminiviridae)	Índia	Mosca branca ( <i>Bemisia tabaci</i> )	SUDHAKAR RAO et al., 1980
<i>Cowpea chlorotic mottle virus</i> (CCMV)	<i>Bromovirus</i> (Bromoviridae)	Estados Unidos	Besouros ( <i>Cerotoma arcuata</i> )	KUHN; DEMSKI, 1987
<i>Cowpea mild mottle virus</i> (CPMMV) [Groundnut crinkle virus]	<i>Carlavirus</i> (Betaflexiviridae)	China, Índia, Indonésia, Costa do Marfim, Nigéria, Tailândia, Filipinas, Papua Nova Guiné, Sudão e Quênia	Mosca branca e semente	DUBERN; DOLLET, 1981; SIVAPRASAD; SREEVINASULU, 1996;
<i>Cucumber mosaic virus</i> (CMV)	<i>Cucumovirus</i> (Bromoviridae)	China	Semente e afídeo	XU; BARNETT, 1984
<i>Peanut stunt virus</i> (PSV)	<i>Cucumovirus</i> (Bromoviridae)	Sudão, Japão, Espanha e Estados Unidos	Mecânica, afídeo e semente	MILLER; TROUTMAN, 1966; FISHER; LOCKHART, 1978
<b><i>Tobacco streak virus</i> (TSV)</b>	<b><i>Irlavirus</i> (Bromoviridae)</b>	<b>Brasil e Índia</b>	<b>Mecânica e polén</b>	<b>COSTA; CARVALHO, 1961</b>
<i>Groundnut rosette assistor virus</i> (GRAV)	<i>Luteovirus</i> (Luteoviridae)	Toda África e sul do Sahara	Afídeo	HULL; ADAMS, 1968; REDDY et al., 1985; NAIDU et al., 1999
<i>India peanut clump virus</i> (IPCV)	<i>Peduvirus</i> (Virgaviridae)	Índia e Paquistão	Mecânica, fungo e semente	REDDY et al., 1983; NOLT et al., 1988
<i>Peanut clump virus</i> (PCV)	<i>Peduvirus</i> (Virgaviridae)	Nigéria, Burquina Faso, Costa Rica e Senegal	Mecânica, fungo e semente	THOUVENEL et al., 1976
Groundnut chlorotic spotting virus (GCSV)	<i>Potexvirus</i> (Virgaviridae)	Costa Rica	Mecânica	DOLLET et al., 1987
<i>Bean yellow mosaic virus</i> (BYMV)	<i>Potyvirus</i> (Potyviridae)	Estados Unidos	Mecânica e afídeo	BAYS; DEMSKI, 1986
<b><i>Cowpea aphid-borne mosaic virus</i> (CABMV)</b>	<b><i>Potyvirus</i> (Potyviridae)</b>	<b>Brasil</b>	<b>Semente e afídeo</b>	<b>PIO-RIBEIRO et al., 2000</b>
<i>Groundnut eyespot virus</i> (GEV)	<i>Potyvirus</i> (Potyviridae)	Costa Rica	Mecânica e afídeo	DUBERN & DOLLET, 1980
<i>Peanut chlorotic blotch virus</i> (PCBV)	<i>Potyvirus</i> (Potyviridae)	África do Sul	Mecânica e afídeo	COOK et al., 1998
<i>Peanut green mosaic virus</i> (PGMV)	<i>Potyvirus</i> (Potyviridae)	Índia	Mecânica e afídeo	SREENIVASULU et al., 1981
<i>Peanut green mottle virus</i> (PeGMoV)	<i>Potyvirus</i> (Potyviridae)	Índia	Nenhuma informação encontrada	SREENIVASULU et al., 1981
<b><i>Peanut mottle virus</i> (PeMoV),</b>	<b><i>Potyvirus</i> (Potyviridae)</b>	<b>No mundo todo</b>	<b>Mecânica, afídeo e</b>	<b>KUHN, 1965; BEHNCKEN, 1980;</b>



[Peanut mild mosaic], [Peanut severe mosaic], [Groundnut mottle virus]			semente	RAJESWARI et al., 1983
<i>Peanut stripe virus</i> (PStV)	<i>Potyvirus</i> ( <i>Potyviridae</i> )	<b>Brasil, China, Índia, Indonésia, Japão, Malásia, Coréia, Filipinas, Myanmar, Tailândia, Taiwan, Vietnã e Estados Unidos</b>	Mecânica, afídeo e semente	DEMSKI et al., 1988; XU et al., 1983; HONG-SOO et al., 2006
<i>Passion fruit woodiness virus</i> (PWV)	<i>Potyvirus</i> ( <i>Potyviridae</i> )	Austrália	Mecânica e afídeo	BOSWEL; GIBBS, 1983
Groundnut veinal chlorosis virus (GVCV)	<i>Rhabdovirus</i> – gênero não atribuído ( <i>Rhabdoviridae</i> )	Índia e Indonésia	Nenhuma informação encontrada	NAIDU et al., 1989
<i>Peanut chlorotic streak virus</i> (PCSV)	<i>Soymovirus</i> ( <i>Caulimoviridae</i> )	Índia	Mecânica	REDDY et al., 1993
Peanut chlorotic streak virus-Vein Banding Strain (PCSV-VB)	<i>Soymovirus</i> ( <i>Caulimoviridae</i> )	Índia	Mecânica	SATYANARAYANA et al., 1994
<i>Groundnut bud necrosis virus</i> (GBNV) [Peanut bud necrosis virus]	<i>Tospovirus</i> ( <i>Bunyaviridae</i> )	Índia, Nepal, Sri Lanka, China, Taiwan, Indonésia e Tailândia	Trips	GHANEKAR et al., 1979
<i>Groundnut chlorotic fan-spot virus</i> (GCFSV)	<i>Tospovirus</i> ( <i>Bunyaviridae</i> )	Taiwan	Mecânica e trips	CHU et al., 2001
<b><i>Groundnut ringspot virus</i> (GRSV)</b>	<b><i>Tospovirus</i> (<i>Bunyaviridae</i>)</b>	<b>Ámerica do Sul e África</b>	<b>Mecânica e trips</b>	<b>PETERS, 2003;</b>
<i>Ground yellow spot virus</i> (GYSV) [Peanut yellow spot virus]	<i>Tospovirus</i> ( <i>Bunyaviridae</i> )	Índia e Tailândia	Mecânica e trips	SATYANARAYANA et al., 1996
<i>Impatiens necrotic spot virus</i> (INSV)	<i>Tospovirus</i> ( <i>Bunyaviridae</i> )	Estados Unidos	Mecânica e trips	PAPPU et al., 1999
<b><i>Tomato spotted wilt virus</i> (TSWV)</b>	<b><i>Tospovirus</i> (<i>Bunyaviridae</i>)</b>	<b>América do Norte, América do Sul e Nigéria</b>	<b>Mecânica e trips</b>	<b>SAMUEL et al., 1930</b>
<i>Peanut yellow mottle virus</i> (PeYMV)	<i>Tymovirus</i> ( <i>Tymoviridae</i> )	Nigéria	Mecânica e besouro	LANA, 1980
<i>Groundnut rosette virus</i> (GRV)	<i>Umbravirus</i> ( <i>Tombusviridae</i> )	Toda a África, sul do Sahara	Mecânica e afídeo	NAIDU et al., 1999; TALIANSKY et al., 2000
Sunflower yellow blotch virus (SuYBV)	<i>Umbravirus</i> ( <i>Tombusviridae</i> )	Malawi, Quênia, Zâmbia e Tanzânia	Mecânica e afídeo	THEURI et al., 1987

\*Em azul, espécies virais que já foram relatadas no Brasil

### **3. Fontes de variabilidade genética em vírus**

A variabilidade genética é gerada por erros que ocorrem durante a replicação dos genomas. Diversos são os processos responsáveis por gerar variabilidade genética dentro de uma população viral, entre tais processos estão: mutações, recombinação e pseudo-recombinação. As mutações são alterações no genótipo dos organismos como as substituições, deleções, inserções, inversões de nucleotídeos, dentre outros, que são transmitidas a partir de uma geração parental para seus descendentes (GÁRCIA-ARENAL; FRAILE; MALPICA, 2003). A recombinação é o processo em que regiões de segmentos com informação genética são trocados entre fitas de nucleotídeos de diferentes haplótipos durante o processo de replicação, resultando em intercâmbio gênico. Já a pseudo-recombinação é o mecanismo de troca de segmentos entre vírus distintos (GÁRCIA-ARENAL; FRAILE; MALPICA, 2003). Análises populacionais de vários vírus de DNA e RNA evidenciam que a recombinação pode ser a maior fonte de variação para que ocorra a evolução (PADIDAM et al., 1999; WOROBEY; HOLMES, 1999).

A frequência dos processos de replicação, as taxas de ocorrência de co-infecções, o modo de transmissão, o tamanho e a estrutura das populações virais e de hospedeiros são fatores que influenciam a geração da variabilidade genética viral. Quando os vírus se replicam no interior de uma célula, o material genético viral pode sofrer mutações, originando uma grande diversidade genética a partir de um único tipo de vírus (BOERLIJST; BONHOEFFER; NOWAK, 1996).

### **4. Aspectos gerais sobre os métodos de detecção e caracterização de vírus de planta**

A identificação precisa do agente causal de uma doença é um pré-requisito essencial para a recomendação de medidas de manejo. Nas últimas décadas diversas técnicas vêm sendo elaboradas como utilização de plantas indicadoras, as técnicas sorológicas e moleculares (DANIELS, 1999; ZERBINI; ALFENAS-ZERBINI, 2007). Apesar do desenvolvimento contínuo de novos métodos mais sofisticados para a detecção de vírus, poucos são adotados para uso rotineiro em laboratórios de Virologia Vegetal, a exemplos do teste de imunoadsorção com enzima ligada ao anticorpo (ELISA), a reação em cadeia da polimerase (PCR) e hibridização de ácidos nucleicos.

Devido ao seu pequeno tamanho e pouca variação morfológica os vírus em geral não podem ser detectados pelos métodos utilizados para outros agentes fitopatogênicos. Por essas características, ainda há certa dificuldade na detecção viral, e em se conhecer a sua diversidade genética. Atualmente acredita-se que menos de 1% da diversidade viral existente seja conhecida (MOKILI; ROHWER; DUTILH,2012). Uma nova abordagem como a metagenômica aliada aos avanços nas tecnologias de sequenciamento, como as de alto desempenho ou sequenciamento de nova geração (*Next Generation Sequencing* - NGS) vem proporcionando uma ampla exploração do universo dos microrganismos, permitindo a sua análise e caracterização em diversas áreas da ciência, incluindo a Virologia Vegetal.

### **5. Sequenciamento de nova geração (*Next Generation Sequencing* - NGS)**

As novas tecnologias de sequenciamento referidas como NGS começaram a ser utilizadas em 2005 (CARVALHO; SILVA, 2010). São tecnologias que permitem acelerar e baixar o custo do processo de sequenciamento. Existem diferentes sequenciadores de NGS, porém todos se baseiam no processamento paralelo massivo de fragmentos de DNA. A grande diferença entre o sequenciador de eletroforese e os sequenciadores da nova geração, consiste na quantidade de fragmentos de DNA processados. Enquanto que um sequenciador de eletroforese processa, no máximo, centenas de fragmentos por vez, os sequenciadores da nova geração podem ler até bilhões de fragmentos ao mesmo tempo (VARUZZA, 2013). Uma das principais vantagens destas plataformas é a capacidade de determinar dados de sequências a partir de fragmentos de DNA de uma biblioteca, não necessitando a realização de clonagem com vetores plasmidiais para a aquisição da sequência (BARZON et al., 2013).

O NGS se refere às plataformas que possuem a capacidade de produzir grandes quantidades de *reads* (fragmentos sequenciados de DNA) com tamanho de 25 e 400 bp. Esses *reads* são menores do que os gerados no sequenciamento tradicional de Sanger, que podem gerar *reads* com sequências de 300 a 750 pb. Dentre as novas plataformas de sequenciamento duas já possuem ampla utilização em todo o mundo: a plataforma 454 FLX da Roche e Illumina. Entretanto, mais dois sistemas de sequenciamento começaram a ser utilizados, que são as plataformas da *Applied Biosystems*, que são denominadas SOLiD Systems, e o *Heliscope True Single Molecule Sequencing* (tSMS), da Helicos (CARVALHO; SILVA, 2010).

Essas plataformas possuem diferenças bioquímicas, de protocolos de sequenciamento, rendimento e tamanhos de sequências produzidas (METZKER, 2010; BARZON et al., 2011). Deste modo, a plataforma SOLiD system, que se caracteriza por possuir alto rendimento mas gera pequenos *reads*, sendo provavelmente mais adequada para aplicações tais como grandes projetos de sequenciamento de genomas maiores ou projeto de sequenciamento de RNA. Em compensação, outras plataformas como a 454 da Roche e Illumina podem fornecer dados adequados para a montagem *de novo*. Dentre os fatores mais importantes na seleção de uma plataforma de sequenciamento incluem o tamanho do genoma a ser estudado, e o teor de G + C (MASSART, 2014). As funções dos programas para análise de dados do NGS podem ser divididas em quatro categorias que incluem alinhamento de sequência dos *reads*, detecção de polimorfismos, montagem de genomas e anotação gênica. Novas ferramentas de programa para análise de sequenciamento de pequenos *reads* estão sendo desenvolvidas continuamente em todo o mundo, especialmente nos Estados Unidos, Europa e Austrália (BARBA; CZONESK; HADIDI, 2014).

As aplicações biológicas do NGS são focadas principalmente no sequenciamento completo do genoma de um organismo, tais como os seres humanos, primatas, cães, gatos, ratos, nematoides, bactérias, fungos, vírus, entre outros (BARBA; CZONESK; HADIDI, 2014). Entretanto, existe um grupo de aplicações que é mais utilizado pela comunidade científica, dentre elas estão: ressequenciamento genômico, *Target sequencing*, RNA seq, Sequenciamento *de novo* e a Metagenoma (VARUZZA, 2013).

Em metagenômica, a aplicabilidade do NGS está no estudo das comunidades de microrganismos diretamente de seus ambientes naturais, sem necessitar de um isolamento dos mesmos em meio de cultivo. O termo metagenômica foi utilizado pela primeira vez por Handelsman et al. (1998), que realizaram o estudo de genomas de várias espécies de microrganismos presentes em uma amostra ambiental extraída do solo. Existem dois tipos de estudo de metagenomas: o da diversidade, utilizando o gene ribossomal 16S e o *Shotgun Metagenomics*, no qual não se faz seleção da sequência alvo, e todo o DNA é extraído da amostra, fragmentado e sequenciado. Esta análise consiste em montar o metagenoma da amostra para tentar a identificação e diversidade de genomas e novos genes (SCHOLZ; LO; CHAIN, 2012).

## 6. Sequenciamento de nova geração aplicada à Virologia Vegetal

Essas novas tecnologias de sequenciamento combinada com a bioinformática sofisticada vêm recentemente contribuindo para avanços significativos nas várias áreas da virologia, principalmente na descoberta de vírus ainda não conhecidos. O primeiro trabalho no campo virológico utilizando a metagenômica foi realizado por Breitbart et al. (2002), evidenciando elevada abundância de vírus em ambiente marinho. Na Virologia Vegetal, os estudos de metagenômica se iniciaram em 2009 (AL RWAHNIH et al., 2009), amplificando o nível de conhecimentos desta ciência, principalmente nas áreas de sequenciamentos de genomas, ecologia, descobertas de novas espécies e gêneros de vírus, epidemiologia, transcriptomas, replicação, detecção e identificação. Devido à capacidade de utilizar extrações de RNAs, o uso de NGS vem se tornando cada vez mais comum para o sequenciamento completo de genomas de vírus de plantas obtendo-se excelentes resultados. O grande desafio dessa técnica, não reside no acesso e utilização da tecnologia, mas na análise e interpretação da grande quantidade de dados, que se tornam disponíveis (KEHOE et al.; 2014). As análises utilizando estas técnicas para vírus ou viroides existentes em amostras biológicas ou ambientais são denominadas de "viroma" (BARBA; CZONESK; HADIDI, 2014).

Recentemente um pequeno número de vírus de RNA de plantas e viroides foram identificados a partir de tecidos infectados e sequenciados por RNA-seq (Illumina). Qualquer ácido nucleico ou RNA de cadeia dupla (dsRNA) extraído a partir do tecido vegetal infectado pelo patógeno, seja vírus ou viroide, podem ser identificados por NGS.

Existem outras sequências virais estudadas por NGS, como a de sequenciamento de *small interfering RNAs* (siRNAs), que estão presentes em todos os eucariotos infectados. Em resposta à ação de viroides ou vírus de RNA ou DNA, a planta hospedeira gera moléculas de siRNA específicas, de 21 a 24 nt de comprimento. A interferência de RNA (RNAi) é um sistema de vigilância citoplasmática para reconhecer dsRNA e especificamente destruir moléculas de RNA de cadeia simples e duplas homólogas ao indutor, usando pequenos RNAs interferentes (siRNA) como um guia (KEHOE et al., 2014). Além disso, o sequenciamento de siRNAs oferece boas oportunidades para identificar agentes de doenças de plantas em infecções assintomáticas, incluindo os vírus ou viroides ainda não conhecidos (MASSART, 2014; BARBA; CZONESK; HADIDI, 2014; KREUZE, 2014).

Amostras ambientais podem ser submetidas a procedimentos prévios, como o enriquecimento de partículas virais, antes da obtenção de ácidos nucleicos. Para tanto, podem ser usadas centrifugação ou filtração seguida de extração direta de ácidos nucleicos (RNA e/ou DNA). Hall et al. (2014) realizaram avaliações de técnicas rápidas e simples para enriquecimento de partículas virais antes da análise metagenômica. Estes mesmos autores em um estudo sobre métodos simples de enriquecimento, cinco combinações de três métodos foram selecionadas e realizadas, pelas etapas de centrifugação, filtração seguido de tratamento com nucleases (DNAses e RNAses).

Vários métodos para otimização e garantia de qualidade e quantidade de ácidos nucleicos vem sendo desenvolvidos, dentre esses, podem ser citados a extração de siRNA (small RNA), a extração de dsRNA (double strand RNA) e o de enriquecimento de partículas virais como no trabalho feito por Silva (2015), em que amostras de plantas forrageiras dos gêneros *Brachiaria*, *Panicum* e *Pennisetum* passaram por um processo de purificação com o objetivo de isolar o máximo de partículas virais icosaédricas e flexuosas. Semelhante a estes estudos, Roossinck et al. (2010) realizaram extração de dsRNA, com amostras de conservação com alta diversidade de espécies vegetais.

A utilização de siRNA no NGS possibilita a identificação de vírus conhecidos e ainda não conhecidos, mesmo estando em uma baixa concentração viral e em infecções assintomáticas. O sequenciamento de alto desempenho fornece milhões de sequências de siRNA de vírus e quando essas sequências são suficientemente abundantes, os fragmentos de vírus gerados podem ser agrupados, possibilitando a montagem completa do genoma de um vírus. Loconsole et al. (2012), através da purificação e construção de uma biblioteca de cDNA de siRNA, e a utilização no sequenciamento por NGS, realizaram a identificação e caracterização do *Citrus yellow vein clearing virus* (CYCV), considerado um novo membro do gênero *Mandarivirus*. O sequenciamento utilizando siRNA possibilita a identificação de muitas espécies já conhecidas e não conhecidas de vírus de plantas, de todos os tipos possíveis de genoma, como também de viroides (KREUZE, 2014).

A maioria dos vírus de plantas possui genoma de RNA e produz formas replicativas de ácido nucleico que são dsRNA. Roossinck et al. (2010) utilizaram dsRNS em estudos metagenômicos com materiais de áreas de conservação e preservação, tendo identificado 344 espécies virais, das quais apenas 30% correspondiam a vírus conhecidos. Nesse estudo, as

plantas amostradas foram submetidas à extração de ácidos nucleicos enriquecidos para dsRNA por meio de cromatografia de celulose CF11, convertidos em cDNA e analisados por multiplexagem e análise da sequência usando pirosequenciamento (ROOSSINCK, 2012).

O uso recente dessas novas tecnologias de sequenciamento na Virologia Vegetal revelou que algumas doenças de etiologia ainda não conhecida, que afetam plantas herbáceas e gramíneas ou infecções latentes em hospedeiros silvestres de diferentes espécies, são causadas por vírus ainda não identificados ou já conhecidos, porém ainda pouco caracterizados. Em torno de 50 artigos já foram publicados descrevendo a identificação de novos vírus de plantas realizados por NGS, em diferentes culturas (PRABHA; BARANWAL; JAIN, 2013; BARBA; CZONESK; HADIDI, 2014). Em batata-doce (*Ipomoea batatas* (L.) Lam.) foram identificados dois vírus de dsDNA (*Badnavirus*) e um vírus ssDNA (*Mastrevirus*) (KREUZE et al., 2009). Um novo *Cucumovirus* foi detectado em *Liatris spicata* (L.) Willd., o qual foi chamado provisoriamente de Gayfeather mild mottle virus (ADAMS et al., 2009). Em pimenta e berinjela (*Solanum melongena* L.) foi detectado a sequência do genoma completo de dois novos vírus denominados de *Pepper yellow leaf curl virus* (gênero *Polerovirus*) e *Eggplant mild leaf mottle* (gênero *Ipomovirus*) (DOMBROVSKY et al., 2011). No tomate, a sequência do genoma completo de uma nova espécie do gênero *Potyvirus* foi detectada, o qual foi denominado de *Tomato necrotic stunt virus* (ToNStV) (LI et al., 2012). Além disso, quatro novas espécies virais, classificadas nos gêneros *Potyvirus*, *Sadwavirus* e *Trichovirus*, foram encontradas em 17 espécies de plantas silvestres na Austrália (WYLIE et al., 2012; BARBA; CZONESK; HADIDI, 2014).

Os vírus *Maize chlorotic mottle virus* (MCMV) e *Sugarcane mosaic virus* (SCMV) foram identificados em folhas de milho com sintomas de necrose letal, doença nova e prejudicial à cultura, registrada pela primeira vez no Quênia em 2011, através do NGS (Roche 454 GS-FLX). Este método foi utilizado para a identificação rápida destes dois vírus em amostras coletadas em campo, obtendo mais de 90% do genoma sequenciado de ambos, permitindo a caracterização o que demonstra elevado potencial do NGS em fornecer um diagnóstico eficiente e rápido (ADAMS et al., 2014). Esta tecnologia, também, tem se mostrado uma poderosa ferramenta para diagnose de doenças de fruteiras de clima temperado (THEKKE-VEETIL et al., 2012), culturas cítricas (LOCONSOLE et al., 2012) e videira (COETZEE et al., 2010).

O último relatório do *International Committee on Taxonomy of Viruses* (ICTV), lista cerca de 3.186 espécies virais (ICTV, 2014) e, segundo Mokili et al. (2012), menos que 1% da diversidade viral é conhecida. Com a utilização do NGS nesta ciência, vai certamente aumentar, de forma significativa, o número de vírus conhecidos, à medida que novas espécies forem sendo descobertas e caracterizadas em diferentes hospedeiros (WYLIE et al., 2012).

A metagenômica juntamente com as tecnologias NGS demonstraram ser sensíveis, precisas e rápidas para a detecção e identificação de sequências virais e de viroides conhecidos e ainda não conhecidos em diferentes espécies de plantas infectadas, incluindo culturas lenhosas perenes, que possuem baixas quantidades destes patógenos. As informações geradas por estas tecnologias podem ser utilizadas de forma eficaz para melhorar a eficiência e confiabilidade dos programas que visam à eliminação de vírus e viroides a partir de material de propagação vegetativa. Sendo assim, o NGS está sendo uma ferramenta de grande importância e poderosa para a detecção e caracterização destes patógenos, como também no auxílio para o manejo de doenças causadas pelos mesmos (ADAMS et al., 2014; BARBA; CZONESK; HADIDI, 2014; MASSART, 2014).

O objetivo deste trabalho foi realizar o estudo do viroma de plantas de amendoim exibindo sintomas típicos de viroses, coletadas em áreas produtoras no Estado de São Paulo.

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**CAPÍTULO II**



**Virome of peanut plants (*Arachis hypogaea*) from São Paulo**

## Virome of peanut plant (*Arachis hypogaea*) from São Paulo

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### Abstract

The peanut (*Arachis hypogaea* L.) is an important oleaginous crop, considered one of the most cultivated plants in Brazil and in the world, mainly as a source of protein and oil. Recently, the metagenomics and the Next Generation Sequencing (NGS) have permitted the exploration of micro-universe in various areas of science, including the Plant Virology. These techniques, combined with bioinformatics, are excellent tools for detecting and characterizing all viral genomes present in a group of samples. The objective of this work was to study virome of peanut plants exhibiting typical virus symptoms, collected in 10 counties of the State of São Paulo by NGS. The metagenomic analyzes detected complete genome sequences of the species *Peanut mottle virus* - PeMoV (genus *Potyvirus*) and *Groundnut ringspot virus* - GRSV (genus *Tospovirus*). Amplification by RT-PCR, using specific primers to the three genomic segments, and Sanger sequencing were also undertaken for a GRSV isolate. The phylogenetic analyzes of the L, M and S segments of tospoviruses proved that the GRSV isolate from peanut, studied in this work, grouped with sequences of an isolate of GRSV from watermelon and a recombinant isolate of GRSV and TCSV from tomato. Furthermore, the phylogenetic analysis of N protein, used for the taxonomic species of tospoviruses, demonstrated that the isolate described here grouped with sequences of GRSV species. It is believed that GRSV and TCSV species share the same segment M. In a further study of the virus isolates, the results of biological analysis (indicator plants) and serological (Dot-ELISA), performed with three selected isolate (A1, N1 e O1), matched those obtained by metagenomic. On the other hand, the sequence analysis of PeMoV coat protein revealed that the Brazilian peanut isolate reported here is phylogenetically related with an isolate from

soybean (*Glycine max* L.), found in South Korea, with 98% identity. This is the first report in Brazil and in the world of the complete genome sequences of PeMoV and GRSV in peanuts, analyzed by high-performance sequencing technologies (NGS).

## Resumo

O amendoim (*Arachis hypogaea* L.) é uma importante oleaginosa, considerada uma das plantas mais cultivadas no Brasil e no mundo, principalmente como fonte de proteína e óleo. Recentemente, a metagenômica e o Sequenciamento de nova geração (*Next Generation Sequencing*-NGS) têm propiciando a exploração do universo de microrganismos em várias áreas das ciências, inclusive na Virologia Vegetal. Estas técnicas, juntamente com a bioinformática são excelentes ferramentas para detecção e caracterização de todos os genomas virais presentes em um determinado grupo de amostras. O objetivo deste trabalho foi estudar viroma de plantas de amendoim exibindo sintomas virais típicos, coletadas em 10 municípios no Estado de São Paulo por NGS. Pela análise metagenômica foi detectado o genoma completo das espécies *Peanut mottle virus*- PeMoV (gênero *Potyvirus*) e *Groundnut ringspot virus*- GRSV (gênero *Tospovirus*). Amplificação por RT-PCR, usando primers específicos para os três segmentos genômicos, e sequenciamento pelo método de Sanger foram também realizados para um isolado de GRSV. Análises filogenéticas para os segmentos L, M e S de tospovírus revelaram que o isolado de GRSV de amendoim estudado neste trabalho se agrupou com sequências de um isolado de GRSV de melancia e um isolado recombinante de GRSV e TCSV de tomate. Além disso, análises filogenéticas com a proteína N, utilizada para a taxonomia de espécies de tospovírus, demonstrou que o isolado de amendoim se agrupou com isolados da espécie GRSV. Em outros estudos dos isolados virais, os resultados das análises biológicas (plantas indicadoras) e sorológicas (Dot-ELISA), realizadas para três isolados selecionados (A1, N1 e O1), coincidiram com os obtidos por metagenômica. Por outro lado, a análise da sequência da capa proteica de PeMoV, revelou que o isolado brasileiro de amendoim relatado nesse trabalho está relacionado filogeneticamente com um isolado obtido de soja (*Glycine max* L.), encontrado na Coreia do Sul, apresentando 98% de identidade. Este é o primeiro relato no Brasil e no Mundo das sequências do genoma completo de PeMoV e GRSV em amendoim, por meio do uso de tecnologias de sequenciamento de alto desempenho (NGS).

## Introduction

Peanut (*Arachis hypogaea* L.) is currently the fourth oilseed crop most widely grown in the world, presenting a high socioeconomic importance in more than 30 countries. China leads the production with 40.8%, followed by India and Nigeria with 14 and 7.5%, respectively [1]. According to recent information from CONAB, the Brazilian production of peanuts is concentrated in the State of São Paulo, with 91.4% of the amount produced, much of which is destined to the fresh market and food industries [2, 3].

Among the factors that affect this oleaginous crop, the viruses cause a reduction in productivity and in the quality of the products. To date, there have been registered 31 viral species, classified into 14 genera, naturally infecting peanut. In Brazil, six species classified into three genera have been reported: *Peanut mottle virus* - PeMoV, *Cowpea aphid-borne mosaic virus* - CABMV and *Bean common mosaic virus*, strain Peanut stripe (BCMV-PSt (genus *Potyvirus*); *Tomato spotted wilt virus* - TSWV and *Groundnut ringspot virus* -GRSV (genus *Tospovirus*) and *Tobacco streak virus*- TSV, (genus *Irlavirus*) [4, 5, 6, 7, 8, 9].

The *Potyvirus*, member of the *Potyviridae* family, is the genus that has a higher number of species that affect peanut crop. These viruses have elongated flexuous particles, with about 680 to 900 nm long and about 11 to 13 nm in diameter. The genome consists of one single stranded RNA (ssRNA) molecule of positive sense, which is surrounded by a coat protein (CP). The RNA of potyviruses connected to a genome-linked protein (VPg) at the 5' end and has a polyadenylated tail at 3' end. The VPg and CP are the only gene products that constitute the viral particle [10, 11, 12].

The criteria for demarcating species within the *Potyviridae* family are: genome showing CP amino acid and nucleotide sequences smaller than 80% and 76% of identity, respectively, and differences in the polyprotein cleavage site, host range, pathogenicity, cytopathology, the transmission mode and serological differences [13].

PeMoV was reported for the first time in 1961, in the United States [14], and is widespread in the producing regions of peanuts. In Brazil, [15] referred to this species as "peanut mosaic". It was detected by Andrade et al. [16] and Nascimento et al. [17], infecting peanuts in the states of São Paulo and Paraíba, respectively. The symptoms in peanuts consist in irregular patches, ranging from mottled mosaic, leaf deformation in the ribs and leaflets, and may also cause drastic reduction of plant and of the pod size [18, 19]

Flasinsky et al. [20] sequenced for the first time the complete genome sequence of PeMoV infecting *Cassia* sp. in the United States. Lim et al. [21] in South Korea, obtained the complete sequence of a PeMoV isolate from soybean, both available in the GenBank. To date, only these two complete sequences of PeMoV have been deposited, none from peanut and Brazil.

The *Tospovirus* genus belongs to *Bunyaviridae* family, which is the largest family of arboviruses (arthropod-borne virus) of RNA with about 350 species described to date [22]. The tospoviruses present enveloped spheroidal particles (80-120 nm in diameter) and genome formed by three segments of ssRNA: S (small), with 2.9 kb, M (medium), with 4.9 kb, both with ambisense orientations and L (large) with 8.9 kb, negative sense [23, 24]. Species of the genus are widely distributed around the world and some species have been reported infecting the peanut crop, as TSWV, GRSV, *Peanut bud necrosis virus* (PBNV), *Peanut yellow spot virus* (PYSV) and the tentative species *Peanut chlorotic fan-spot virus* (PCFSV) [24, 25]. The symptoms caused by these viruses include concentric rings, chlorotic spots, mosaic, leaf distortion, stunting, discoloration, and plant death, as well as, great reduction of the number of pods, the size of kernels and plant production [26].

For long time, virus isolates that today would be identified as GRSV, were considered strains of TSWV. However, works using a combination of biological, serological and molecular methods, have determined differences that permitted a taxonomic classification of species within this genus, based, especially, on the amino acid sequence of the nucleoprotein (N). A new species in the genus *Tospovirus* is identified when the identity of the amino acid sequence of the N protein is less than 90%, compared to the sequences of known viruses [27, 28].

In recent years, many *Tospovirus* species have been identified and their genomes completely sequenced. For examples: Pepper necrotic spot virus - PNSV [29] Soybean necrosis associated virus - SVNaV [30], Hippeastrum chlorotic ring virus - HCRV [31], Bean necrotic mosaic virus - BeNMV [32], Tomato necrotic spot virus - TNSV [58], Lisianthus necrotic ringspot virus - LNRV [33] and Mulberry vein banding associated virus - MBaV [34]. Currently, there are sixteen complete genome sequences of *Tospovirus* in GenBank, and, for the GRSV, just the sequence of a recombinant isolate from Florida GRSV-TCSV ( $L_{\text{GRSV}} - M_{\text{TCSV}} - S_{\text{GRSV}}$ ), obtained from tomato [35].

The second generation sequencing or Next-Generation Sequencing (NGS) is characterized by massive parallel processing and high quantity of DNA fragments. The metagenomics and NGS have permitted the exploration of micro-universe in various areas of science, including the Plant Virology. They are important tools for diagnosis of plant diseases caused by viruses, being able to perform the detection of all the viral genomes that are present in certain group of samples, regardless they are known or unknown different strains or viral species [36, 37, 38, 39].

In tomato, the complete genome sequence of *Potyvirus* species was detected, which was called *Tomato necrotic stunt virus* [37]. In addition, four new viral species, classified in *Potyvirus*, *Sadwavirus* and *Trichovirus* genera were found in 17 species of wild plants in Australia [40, 41]. In pepper and eggplant (*Solanum melongena* L.) were detected the sequence of the complete genome of two new virus called Pepper yellow leaf curl virus (genus *Polerovirus*) and Eggplant mild leaf mottle (genus *Ipomovirus*) [42].

More than 50 articles have been published describing the identification of new plant viruses performed by NGS in different crops [38, 41], none of them belonging to Fabaceae family, such as peanuts.

The main objective of this work was to study by NGS the virome of peanut plants collected from producing areas of peanut in São Paulo State and characterize the detected virus isolates through biological, serological and molecular analyzes.

## **Materials and Methods**

This work was carried out at the Laboratory of Virology of the Department of Plant Pathology of the Universidade de Brasília - UnB and in greenhouse and insectary of the Estação Experimental de Biologia (EEB).

### **Virus isolates obtainment and maintenance**

Thirty-three peanut plants with typical virus symptoms from 18 commercial fields in 10 counties of the state of São Paulo were collected in the years 2013/14 (Table 1).

**Table 1. Peanut samples\* collected in commercial fields from different counties of the State of São Paulo**

Counties	Field Code	Identification of samples
Santa Adélia	A	A1, A2
	B	B1, B2
	C	C1, C2
Luzitânia	D	D1
Jaboticabal	E	E1, E2
	F	F1, F2
	G	G1, G2
Itápolis	H	H1, H2
Pindorama	I	I1, I2
Tupã	K	K1
	L	L1, L2
Rancharia	M	M1, M2
Tupã	N	N1, N2
	O	O1, O2
	P	P1, P2
Marília	P	P1, P2
Guainbê	Q	Q1, Q2
	R	R1
Guarantã	S	S1, S2

\* Each sample corresponded a plant exhibiting virus like symptoms

Initially, scions from field materials were grafted on 20 days old peanut plants under greenhouse conditions at the Universidade Federal Rural de Pernambuco (UFRPE). The symptoms were observed on around 20 days after inoculation. Then, symptomatic materials were sent for analyzes at UnB. The maintenance of infected materials was done by successive passages to new peanut plants by grafting.

### **Semi-purification and total RNA extraction**

Prior the total RNA extraction, three groups of leaf tissue samples showing symptoms, 10g each, were semi-purification by macerating in liquid nitrogen and addition of 100 mL of 0.1 M sodium phosphate buffer. Each group of macerated tissue, was filtered on gauze, transferred into 50mL falcon tubes, and centrifuged at 3.800 rpm for 20 min at 4° C. Then, well defined supernatant volume was transferred to ultracentrifuge tubes on a cushion consisting of 20 mL of a 20% sucrose solution added to the tube bottom, and the weight balancing of all tubes was obtained by addition or removal of aliquots of the supernatant. Shortly thereafter, one ultracentrifugation at 33,000 g for 2 hours at 4° C was conducted, and the supernatant carefully discarded, leaving the tubing with the pellets, allowed to dry on the bench.



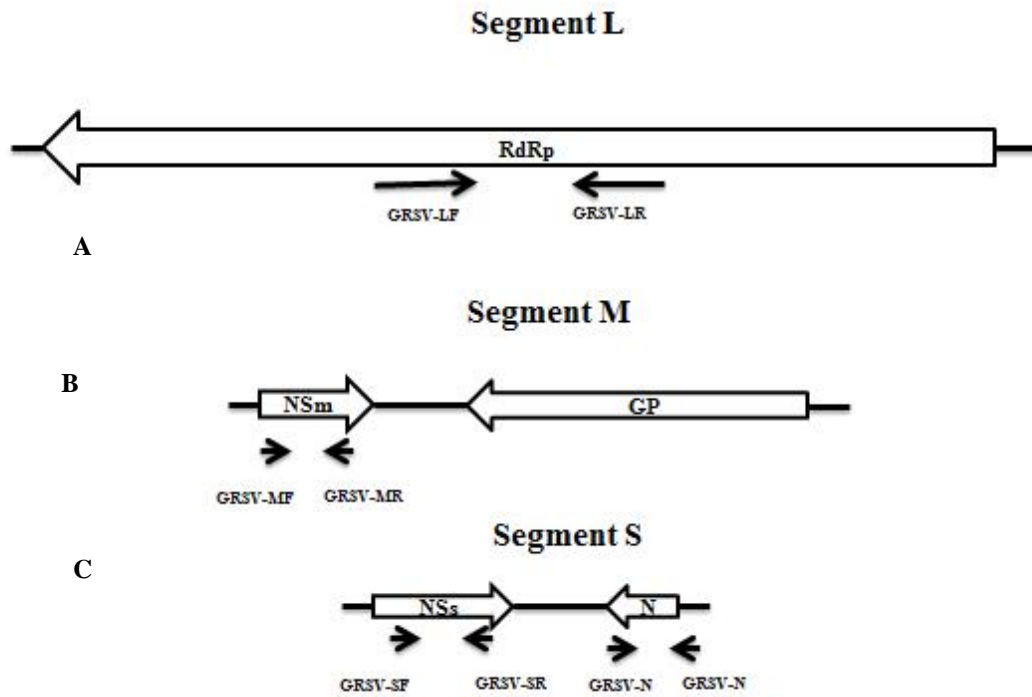
The tubes with pellets were used for RNA extraction by Trizol method, following an optimized protocol for this reagent. After extraction, the total RNA was quantified in Nano Vue and 30 uL (100 µg /uL) were put on RNA stable, which is protected by a gel film which keeps RNA integrity. Then, the tubes were involved in aluminum foil to protect from light, and stored at room temperature until sending for sequencing.

### **Next-Generation Sequencing (NGS), metagenomic and phylogenetic analysis**

Sequencing was performed by Illumina Miseq platform system at Universidade Católica de Brasília. The sequenced fragments (*Reads*) were analyzed CLC Genomics Workbench 8.0 program, where the *contigs* were assembled. The assembled *contigs* were analyzed against the BLAST X program viral RefSeq database (GenBank) and *contigs* related plant viruses were selected. The analyzes of the detected genomes were performed in Geneious 7.1 program.

From the generated sequences, after the detection and identification of the complete genome of PeMoV and GRSV, specific primers were designed in Vector NTI program and synthesized (Fig 1; Table 2).

Each 33 samples used in this study was analyzed for GRSV detection. Phylogenetic analyzes were performed by the maximum Likelihood method, Tamura-Nei model [43], with bootstrap 1.000 replications available in Geneious 7.1 program with MAFFT program. For phylogenetic analyzes, both complete genome sequences of nucleotides and amino acids were used. For GRSV complete virus genome sequences of all segments *Tospovirus* species were used, making the building trees for the L segments, M segment (NSm and GP ) and S segment (NSs and N). The PeMoV analysis was based on complete sequences of the coat protein (CP) available. To build the trees sequences from GenBank listed in Tables 3 and 4 were downloaded.



**Fig 1: Illustration of *Groundnut ringspot virus* - GRSV genome and the position of primers.** The arrows indicate primer annealing positions in different segments (L: large; M: medium and S: small). (A) Primers for L segment flank, amplifying an RNA polymerase region RNA-dependent (RdRp) of approximately 698 base pairs, (B) Primers for M segment flanks, amplifying a fragment of 353 bp NSm about the region, (C) Primers for the segment S flank, amplifying a 502 bp fragment of approximately of region NSs and primer N ORF complete.

**Table 2: Pairs of primers used to detect *Groundnut ringspot virus* - GRSV by primers synthesized from the sequences obtained by amplification of the gene and metagenomic N**

Virus/ Specification	Forward and Reverse Primers	Expected amplicon	Annealing temperature	References
1-GRSV- L Segment	5' AACAGGATTCAGCAATATGG 3' / 5' AATTCCTTGAAGACAATTGTGT 3'	698 pb	54 °C	Synthesis from metagenomic analyses
2-GRSV- M Segment	5' TTTGTCCAACCATACCAGACCC 3' / 5' GGCTTCAATAAAGGCTTGGG 3'	353 pb	59 °C	Synthesis from metagenomic analyses
3-GRSV-S Segment	5' TTCAAACTCAGTTGTACTCTGA 3' / 5' TTACTTTCGATCTGGTTGAA 3'	502 pb	54 °C	Synthesis from metagenomic analyses
4-GRSV- N Gene	5' TATGTCTAAGGTC AAGCTCACAAAAGAAAACAT 3' / 5' CTCATGCAACACCAGCAATCTTGGCTTCTTT 3'	777 pb	56-58 C°	Available by Dr. Renato Resende

**Table 3: Access numbers and acronyms of viruses of the *Tospovirus* genus available in GenBank**

<b>Virus</b>	<b>Acronyms</b>	<b>S RNA</b>	<b>M RNA</b>	<b>L RNA</b>	<b>Locality</b>
Alstromeria necrotic streak virus	ANSV	GQ478668	-	-	Colombia
Bean necrotic mosaic virus	BeNMV	NC_018071	JN587269	NC_018070	Brazil
Calla lily chlorotic spot virus	CCSV	AY867502	FJ822961	FJ822962	Taiwan
Capsicum chlorosis virus	CaCV	NC_008301	NC_008303	NC_008302	Thailand
Chrysanthemum stem necrosis virus	CSNV	NC_027719	KM114547	KM114546	Japan
<i>Groundnut bud necrosis virus</i>	GBNV	NC_003619	U42555	NC-003614	South Asia
Groundnut chlorotic fan-spot virus	GCFSV	AF080526	-	-	Taiwan
<i>Groundnut ringspot virus</i> – SA-05 (South African isolate)	GRSV-SA05	AF487516.1 (N) JN571117.1 (NSs)	-	-	South Africa
<i>Groundnut ringspot virus</i> -TO (Brasil)	GRSV-TO	Available data by Mello, F. L.	Available data by Mello, F. L.	Available data by Mello, F. L.	Brazil
<i>Groundnut ringspot virus</i> -reassortant (South Florida)	GRSV-TCSV reassortant	NC_015467	NC_015468	NC_015469	United State
<i>Groundnut yellow spot virus</i>	GYSV	AF013994	-	-	India
Hippeastrum chlorotic ringspot virus	HCRV	JX833564	JX833565	HG763861	China
<i>Impatiens necrotic spot virus</i>	INSV	NC_003624	DQ425095	NC_003625	China
<i>Iris yellow spot virus</i>	IYSV	AF0011387	FJ361359	FJ623474	United State
Lisianthus necrotic ringspot virus	LNRV	AB852525	-	-	Japan
Melon severe mosaic virus	MSMV	EU275149	-	-	Mexico
Melon yellow spot virus	MYSV	NC_008300	AB061773	NC_008306	Japan
Mulberry vein banding associated virus	MVBaV	KM819701	KM819699	NC_026617	China
Pepper necrotic spot virus	PNSV	HE584762	-	-	Peru
Physalis silver mottle virus	PhySMV	AF067151	-	-	Thailand
<i>Polygonum ringspot virus</i>	PolRSV	KJ81744	KJ541745	KJ541746	Italy
Soybean vein necrosis associated virus	SVNaV	HQ728387	HQ728386	HQ728385	United States
<i>Tomato chlorotic spot virus</i>	TCSV	KP172480 (N)	AY574054.1(GPs) AF213674.1(NSm)	HQ700667	Brazil
<i>Tomato chlorotic spot virus</i>	TCSV-RD	Available data by Mello, F. L.	Available data by Mello, F. L.	Available data by Mello, F. L.	Dominican Republic
Tomato necrosis virus	TNeV	AY647437	-	-	Thailand
Tomato necrotic ringspot virus	TNRV	FJ946835	FJ947152	-	Thailand
Tomato necrotic spot virus	TNSV	KM355773	-	-	China
<i>Tomato spotted wilt virus</i>	TSWV	NC_002051	JN664253	NC_002052	United States
Tomato yellow ring virus	TYRV	AY686718	JN560177	JN560178	Iran
Tomato zonate spot virus	TZSV	NC_010489	EF552434	NC_010491	China
<i>Watermelon bud necrosis virus</i>	WBNV	GF584184	GU584185	GU735408	India
<i>Watermelon silver mottle virus</i>	WSMoV	NC_003843	DQ157768	NC_003832	China
Zucchini lethal chlorosis virus	ZLCV	AF067069 (N)	-	-	Brazil

**Table 4: Access numbers and acronyms of *Peanut mottle virus* - PeMoV isolates used in the phylogenetic analysis**

Acronyms	Hosts	Locality	Access Numbers
PeMoV-Strain M	<i>Cassia</i> sp.	United States	AF023848
PeMoV-M	Pea ( <i>Pisum sativum</i> L.)	United States	BCMCOATCA
PeMoV-PV4	Pea ( <i>Pisum sativum</i> L.)	United States	BCMCOATDA
PeMoV-AR	Pea ( <i>Pisum sativum</i> L.)	United States	BCMCOATAA
PeMoV-3b8	Pea ( <i>Pisum sativum</i> L.)	United States	BCMCOATBA
PeMoV-T	Pea ( <i>Pisum sativum</i> L.)	United States	BCMCOATE
PeMoV-IR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	JX441319
PeMoV-133	Peanut ( <i>Arachis hypogaea</i> L.)	Australia	X73422
PeMoV-DG13	Soybean ( <i>Glycine max</i> L.)	South Korea	KJ664838
PeMoV-Habin	Soybean ( <i>Glycine max</i> L.)	South Korea	KF977830
PeMoV-Gn-Hyd-1	Peanut ( <i>Arachis hypogaea</i> L.)	India	JX08812
PeMoV-Israel	Peanut ( <i>Arachis hypogaea</i> L.)	Israel	DQ868539
PeMoV- QD5	Peanut ( <i>Arachis hypogaea</i> L.)	China	GQ180067
PeMoV- QD6	Peanut ( <i>Arachis hypogaea</i> L.)	China	GQ180068

### Extraction of total RNA from single peanut samples and use in RT-PCR

For further study on the presence of GRSV in the peanut fields, detected by NGS in combined samples, the extraction of total RNA from the 33 samples, individually, was carried out using modified hot-phenol protocol [44], and used in RT-PCR analysis. For amplification of the genome fragments, the protocols recommended by the manufacturer (Invitrogen) were followed.

The cDNA was synthesized using MMLV-RT enzyme from the reverse primers specific for different segments for all segments GRSV using the respective reverse primers (primers here called 1, 2 and 3) are listed in Table 2. The reverse transcription reaction was performed using 10  $\mu$ L at a concentration of 1-2  $\mu$ g of total RNA, 1  $\mu$ L of dNTP (10 mM) and 1  $\mu$ L of reverse primer (10 pmoles/ $\mu$ L), followed by 5 min incubation at a temperature of 70° C to open the stranded RNA. Thereafter was added 4 $\mu$ L 5X MMLV RT buffer, 1  $\mu$ L DTT (0.1 M), 1  $\mu$ L of RNase Out (40U/ $\mu$ L), 1  $\mu$ L of MMLV-RT (200 U/ $\mu$ L) and 1  $\mu$ L H<sub>2</sub>O milliQ, incubation temperature used in this step was 37° C for 60 minutes, followed by an incubation at 70° C for 15 minutes to inactivate the enzyme.

The Platinum Taq DNA Polymerase Invitrogen™ (5U/ $\mu$ L) was used for PCR. The specific primers used in the PCR were 1, 2 and 3 listed in Table 2. The PCR conditions used to amplify regions of each segment GRSV these viruses were: Primers 1 and 2: initial denaturation of 95° C for 3 minutes, denaturation of 94° C for 30 seconds, 54° C to annealing for 45 seconds, extension to 72° C for 1 minute and a final extension to 72° C for 10 minutes

and waiting 12° C, with a total of 35 cycles. Primer 3: initial denaturation 95° C for 3 minutes, denaturation 94° C for 30 seconds, 59° C annealing for 45 seconds, extension 72° C for 1 minute and a final extension 72° C for 10 minutes and waiting 12° C, a total of 35 cycles. The PCR products were applied in agarose gel 1% and subjected to electrophoresis with TBE Running buffer 0.5X, stained in ethidium bromide solution (0.5/mL).

#### **Amplification and purification of the segments L, M and S of *Groundnut virus ringspot virus* - GRSV**

Three isolates of GRSV A1, N1 and O1 were selected and their amplicons obtained with primers 1, 2 and 3 (shown in Table 2) were purified and sequenced. These samples were selected because they had three segments positive to GRSV. After PCR reaction, amplicons of the expected sizes were observed for every segment L, M and S. A new PCR reaction to a final volume of 100 µL was performed and the bands obtained for the different segments were individually eluted using GFX purification kit (GE Healthcare) according to the manufacturer's manual. The samples were stored at -20° C.

#### **Sequencing and phylogenetic analysis of the segments L, M and S *Groundnut ringspot virus* - GRSV**

The purified amplicons of the GRSV isolated A1, N1 and O1 using primers 1, 2 and 3 listed in Table 2, were quantified for 20 ng/µL and the samples sent for the direct sequencing of PCR (Empresa Brasileira de Pesquisa na Agropecuária - EMBRAPA- CNPH). The obtained sequences were compared with sequences available in the BLAST (<http://blast.ncbi.nlm.nih.gov/>).

Phylogenetic analyzes were performed with the Neighbor-Joining method and Tamura-Nei model [43], with 1.000 bootstrap repetitions, the sequences were aligned in the Geneious 7.1 program with the MAFFT program. For phylogenetic analysis of the full genome nucleotide sequences were used for all segments of tospovirus isolates available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The sequences of tospovirus isolates were downloaded from GenBank and listed in Table 3.

#### **RT-PCR using specific primers for amplification of the N gene (segment S) of *Groundnut ringspot virus* - GRSV**

The cDNA for the N gene was synthesized using MMLV-RT enzyme (Invitrogen) according to the manufacturer's recommendations. The GRSV N-F primers and GRSV N-R

(Table 2) were used. The *Platinum Taq DNA Polymerase* Invitrogen™ (5U/uL) was used for the PCR, the conditions used for the PCR amplification of virus GRSV N gene were: initial denaturation 94° C for 30 seconds, 94° C denaturation for 30 seconds, annealing 56° C for 30 seconds, 72° C extension for 1 minute and final extension 72° C for 10 minutes and waiting 12° C, with a total of 35 cycles. The PCR products were applied in agarose gel 1% subjected to electrophoresis with 0.5 TBE running buffer and stained in ethidium bromide solution (0.5/ mL).

#### **Amplicon purification, cloning the N gene of *Groundnut ringspot virus* - GRSV and sequencing by Sanger method**

The GRSV isolates A1, N1 and O1 were selected to have their amplicons purified and sent to sequencing. The bands were eluted from the gel using the GFX purification kit (GE Healthcare). The purified DNA was stored at -20° C. The N gene of isolates A1, N1 and O1 were connected to the input vector for products PCR pGEM®-T Easy (Promega), according to the manufacturer's instructions. The connection between the insert N and pGEM®-T Easy vector, was made with the use of the enzyme T4 DNA ligase (3 U/uL) standing overnight at 4° C. Competent cells of *Escherichia coli* DH5 strain (Invitrogen) were transformed by electroporation according [45]. The recombinant plasmid (pGEM-N) were obtained in accordance with the instructions contained in pGEM®-T Easy vector protocol. The cloned amplicons (A1, N1 and O1) were quantified in approximately 100 ng/uL and submitted for sequencing by Sanger (MACROGEN) with primers T7 promoter and the pUC / M13 reverse. The obtained sequences were compared with sequences available in the BLAST ([http:// http://blast.ncbi.nlm.nih.gov/](http://blast.ncbi.nlm.nih.gov/)) and phylogenetic trees were built.

#### **Phylogenetic trees with sequences of the N gene of *Groundnut ringspot virus* - GRSV**

The sequences were aligned using the program Geneious 7.1 with MAFFT program and the phylogenetic analyzes were performed by Neighbour-Joining method and Tamura-Nei model [43], with 1.000 bootstrap replicates. For phylogenetic analyzes of the complete nucleotide sequences of the N gene obtained in this work, full sequences of GRSV, TCSV e TSWV isolates available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) were used. The sequences of GRSV, TCSV e TSWV isolates were downloaded from GenBank and listed in Tables 5, 6 and 7.

**Table 5: Access numbers of sequences and acronyms of *Groundnut ringspot virus*- GRSV isolates used in phylogenetic analysis of N protein, which are available in GenBank**

Acronyms	Hosts	Locality	Access Numbers
GRSV-13-332	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	KM007032
GRSV-13-331	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	K007031
GRSV-11-219	Gooseberry ( <i>Physalis angulata</i> L.)	United States	KM007030
GRSV-11-189F	Capsicum ( <i>Capsicum annuum</i> )	United States	KM007029
GRSV-11-189D	Capsicum ( <i>Capsicum annuum</i> L.)	United States	KM007028
GRSV-11-189B	Capsicum ( <i>Capsicum annuum</i> L.)	United States	KM007027
GRSV-11-189A	Capsicum ( <i>Capsicum annuum</i> L.)	United States	KM007026
GRSV-11-117	Glossy nightshade ( <i>Solanum americanum</i> Mill)	United States	KM007025
GRSV-11-102	Glossy nightshade ( <i>Solanum americanum</i> Mill)	United States	KM007024
GRSV-Vi	Cucumber ( <i>Cucumis sativus</i> L.)	Brazil	KJ6005652
GRSV-PS8	Peanut ( <i>Arachis hypogaeae</i> L.)	Brazil	KF511799
GRSV-SP1	Peanut ( <i>Arachis hypogaeae</i> L.)	Brazil	KF511798
GRSV-RN7	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49702
GRSV-RN3	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49701
GRSV-T102	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49700
GRSV-T101	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49699
GRSV-M316	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49698
GRSV-M23	Soybean ( <i>Glycine max</i> L.)	Argentina	GRU49696
GRSV-T18	Soybean ( <i>Glycine max</i> L.)	Argentina	GQ455453
GRSV-Arg	Peanut ( <i>Arachis hypogaeae</i> L.)	Argentina	DQ973171
GRSV-SP	Amarillis ( <i>Hippeastrum</i> sp.)	Brazil	AY385780
GRSV-AL	Tobacco ( <i>Nicotiana tabacum</i> L.)	Brazil	AF13219
GRSV-95/0137	Soybean ( <i>Glycine max</i> L.)	South Africa	AF487517
GRSV-95/0188	Soybean ( <i>Glycine max</i> L.)	South Africa	AF487516
GRSV-RJ	Cubiu ( <i>Solanum sessinflorum</i> Dunal)	Brazil	AF251271

**Table 6: Access numbers of sequences and acronyms of of *Tomato chlorotic spot virus* isolates - TCSV used in phylogenetic analysis of N protein, which are available in GenBank**

Acronyms	Hosts	Locality	Access Numbers
TCSV- 15-57	Glossy nightshade ( <i>Solanum americanum</i> Mill)	United States	KR012989
TCSV-15-44	Scarlet eggplant ( <i>Solanum aethiopicum</i> L.)	United States	KR012986
TCSV- Mir-Br07	Garden jalap ( <i>Mirabilis jalapa</i> L.)	Brazil	KP276236
TCSV- 10-10-14	Rosy perinnwinkle ( <i>Catharantus roseus</i> L.)	United States	KP172480
TCSV-14-201	Rosy perinnwinkle ( <i>Catharantus roseus</i> L.)	United States	KP172478
TCSV-14-129	Cactus ( <i>Schlumbergera truncate</i> (Haw) Moran)	United States	KP063318
TCSV-14-41	<i>Hoya wayetii</i> Kloppenb	United States	KP063314
TCSV-OH13	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	KM610235
TCSV-13-33	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	KM007037
TCSV-DRSP1	Pepper ( <i>Capsicum frutescens</i> L.)	Dominican Republic	KJ399304
TCSV-DRF1	Bean ( <i>Phaseolus vulgaris</i> L.)	Dominican Republic	KJ399303
TCSV-13-114	Lettuce ( <i>Lactuca sativa</i> L.)	United States	KF819827
TCSV-DO-2012	Tomato ( <i>Solanum lycopersicum</i> L.)	Dominican Republic	KF420991
TCSV-13-6	<i>Datura stramonium</i> L.	United States	KC969452
TCSV-T36H	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	JX244197
TCSV-T18H	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	JX244196
TCSV-T11MD	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	JX244196
TCSV-96/0742	Tomato ( <i>Solanum lycopersicum</i> L.)	United States	HQ634666
TCSV- Bouvardia	<i>Bouvardia</i> sp.	Brazil	AY380813
TCSV-Jambu	Jambu ( <i>Acmella oleracea</i> (L) R. K. Jansen)	Brazil	AM887766
TCSV-D	<i>Dieffenbachia</i> sp.	Brazil	AF454913
TCSV-SG	gilo ( <i>Solanum aethiopicum</i> L.)	Brazil	AF413110
TCSV-MG	Tomato ( <i>Solanum lycopersicum</i> L.)	Brazil	AF282982

**Table 7: Access numbers of sequences and acronyms of *Tomato spotted wilt virus* - TSWV isolates used in phylogenetic analysis of N protein, which are available in GenBank**

Acronyms	Hosts	Locality	Access Numbers
TSWV-TP-16	Tomato ( <i>Solanum lycopersicum</i> L.)	Turkey	KT192625
TSWV-SC-13	Tomato ( <i>Solanum lycopersicum</i> L.)	Turkey	KT192624
TSWV-YDL-JD3	Cowpea ( <i>Vigna unguiculata</i> L. Walp)	China	KR259539
TSWV-A2	Pepper ( <i>Capsicum frutescens</i> L.)	Argentina	KP719131
TSWV-14YV677	<i>Nicotiana benthamiana</i>	China	KP684518
TSWV-CX1	Tomato ( <i>Solanum lycopersicum</i> L.)	China	KP637174
TSWV-XP	Lettuce ( <i>Lactuca sativa</i> L.)	China	KP330473
TSWV-NRB	Capsicum ( <i>Capsicum annum</i> )	Turkey	KM379142
TSWV-p331	Pepper ( <i>Capsicum frutescens</i> L.)	Italy	KM213989
TSWV-T1081	Tomato( <i>Solanum lycopersicum</i> L.)	Italy	KM096542
TSWV-P302	Pepper ( <i>Capsicum frutescens</i> L.)	Italy	KM96541
TSWV-T992/3	Tomato( <i>Solanum lycopersicum</i> L.)	Italy	KM096540
TSWV-P207	Pepper ( <i>Capsicum frutescens</i> L.)	Italy	KM096539
TSWV-DRT2	Tomato ( <i>Solanum lycopersicum</i> L.)	Dominican Republic	KJ399315
TSWV-PFR11	<i>Hieracium</i> sp.	New Zealand	KC494500
TSWV-Tobacco	Tobacco ( <i>Nicotiana tabacum</i> L.)	United States	HQ406984
TSWV-07W-BerR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406982
TSWV-06G-TomR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406969
TSWV-McC-Tift 3R	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406965
TSWV-06G-CofR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406958
TSWV-McC-BukR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406949
TSWV-GRG-SumR	Peanu ( <i>Arachis hypogaea</i> L.)	United States	HQ406942
TSWV-06G-TerR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406931
TSWV-GNR-ClqR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406926
TSWV-AP4-ClqR	Peanut ( <i>Arachis hypogaea</i> L.)	United States	HQ406920
TSWV-MO	Tomato ( <i>Solanum lycopersicum</i> L.)	Japan	AB921161
TSWV-WT	Tomato ( <i>Solanum lycopersicum</i> L.)	Japan	AB921160

### Virus isolate characterization by host range and Dot-ELISA

To carry out biological analyzes, the three selected peanut samples, previously mentioned, were mechanically inoculated in the following hosts: *Capsicum annum* L., *C. annum* var. IKEDA, *C. annum* var. 679, *Chenopodium amaranticolor* L., *C. quinoa* Wild, *Datura metel* L., *D. stramonium* L., *Gomphrena globosa* L., *Glycine max* L., *Nicotiana benthamiana* Domin., *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. cv. TNN, *Lactuca sativa* L., *Phaseolus vulgaris* L., *Physalis floridana* L., *Solanum lycopersicum* L., e *Vigna unguiculata* L. The inoculation was performed using leaf extracts from infected plants, the concentration of 1:20 (p/v) in 0.01 M phosphate buffer, pH 7.0, containing sodium sulphite and 1% Celite. The symptoms were evaluated at 5, 10, 15, 20, 25 and 30 days after inoculation (DAI), and checking the types of plant reactions, resulting from local and/or systemic infections. On the day of the last assessment, leaf samples from all inoculated plants were collected for Dot-ELISA analysis. This test was undertaken with nitrocellulose membrane Hybond C, in which were applied 2 uL of each sample and used antibodies against the GRSV at concentrations of 1:1000.



## Results

### Maintenance of virus isolates

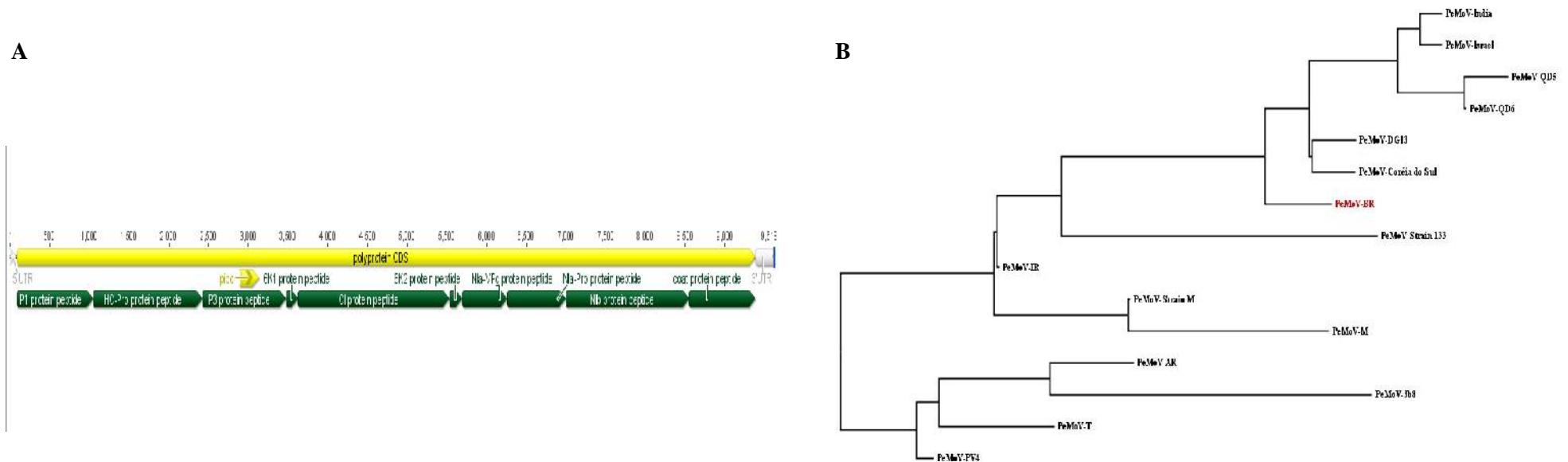
The virus isolates were maintained in peanut plants. Symptoms such as mosaic, chlorotic spots, leaf chlorosis and deformation were observed 20 days after grafting inoculation (Fig 2).



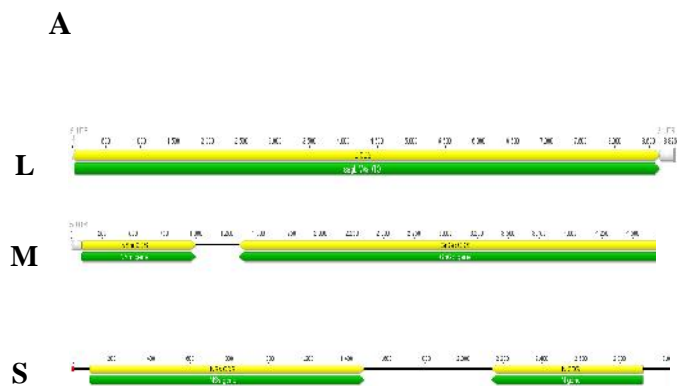
**Fig 2.** Peanut plants (*Arachis hypogaea*) maintained in insectary at Estação Experimental de Biologia (EEB) - Universidade de Brasília (UNB) showing virus symptoms 20 days after inoculation by grafting. Identification of samples in parentheses. (A) Mosaic (N1), (B) Chlorotic points (G2) and (C) Chlorosis and deformation (O1).

### Metagenomic and phylogenetic analyzes

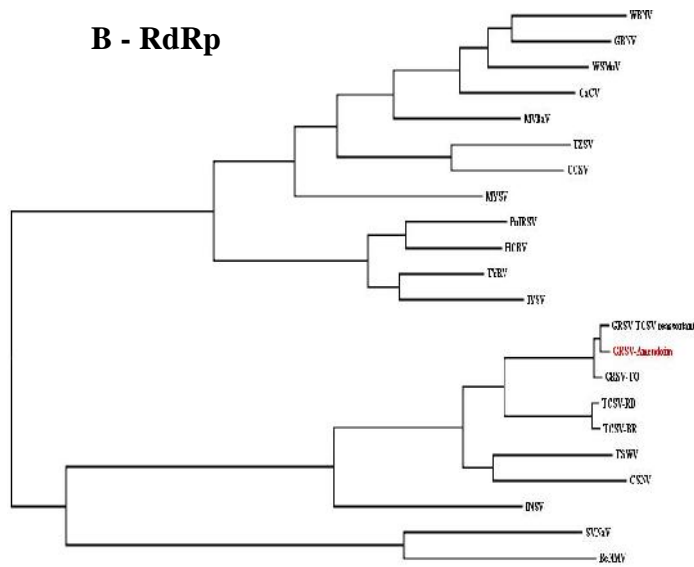
After sequencing performed on the Illumina platform MiSeq 6,727, 862 million of *reads* were obtained and fitting was performed by generating 380 contigs. The consensus generated from the assembled *contigs* detected the complete genome of PeMoV (genus *Potyvirus*) (Fig 3.) with 9,618 nucleotides (nt) and GRSV (genus *Tospovirus*) (Fig 4.), segment L with 8,876 nt, M segment M with 4,925 nt, and the segment S with 3,069 nt. The comparative analysis of the nucleotide sequences of the complete genome of PeMoV and GRSV among genomic sequences of other viruses available in the database (GenBank), revealed that the peanut isolate of PeMoV showed higher nucleotide identity (98%) with a PeMoV isolate obtained from soybean (*Glycine max* L.) from South Korea [21]. The peanut isolate of GRSV showed an identity of 98% for each segment with a recombinant isolate of GRSV and TCSV from tomato (*S. lycopersicum* L.) [35]. Phylogenetic trees constructed with nucleotide sequences of RdRp, NSm, Gn / Gc, NSs and N showed that the GRSV isolated from peanut grouped in the American clade with TCSV and GRSV isolate (Fig. 4).



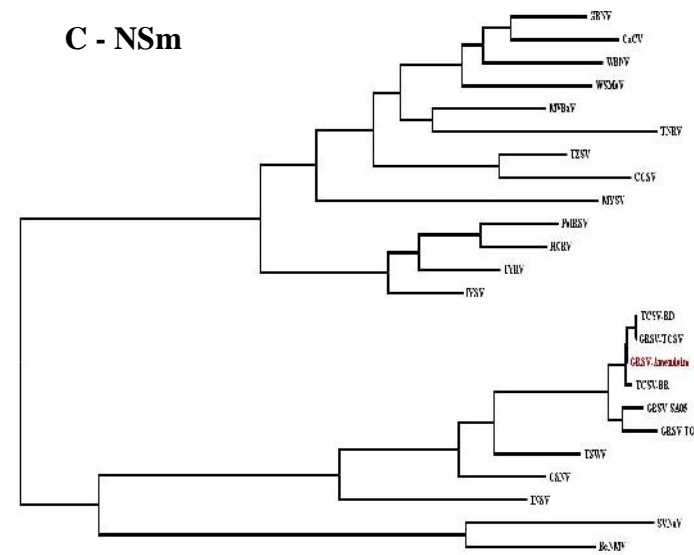
**Fig 3. Schematic representation of the genome organization and phylogenetic position of *Peanut mottle virus* - PeMoV (genus *Potyvirus*) isolated from peanut (*Arachishypogaea*L.) in Brazil (detached in red).** (A) Genomic organization of PeMoV, ORF 1 (Open Reading Frame) encoding for a polyprotein ORF 2 PIPO (Pretty interesting *Potyviridae* ORF) represented in yellow and the other proteins obtained after proteolytic cleavage in green and (B) Phylogenetic tree using amino acid sequences of the coat protein isolated from 14 PeMoV deposited in GenBank. The isolated PeMoV in Brazil is red. The trees were constructed by the maximum likelihood method, model Tamura-Nei with 1000 bootstrap replicates in Geneious 7.1 program.



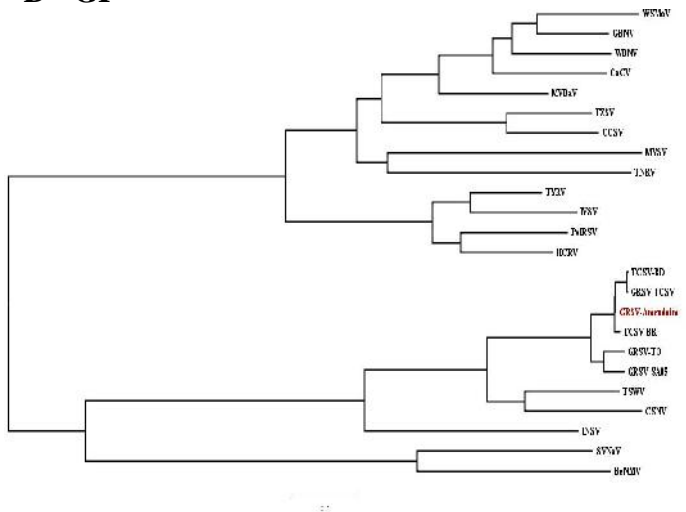
**B - RdRp**



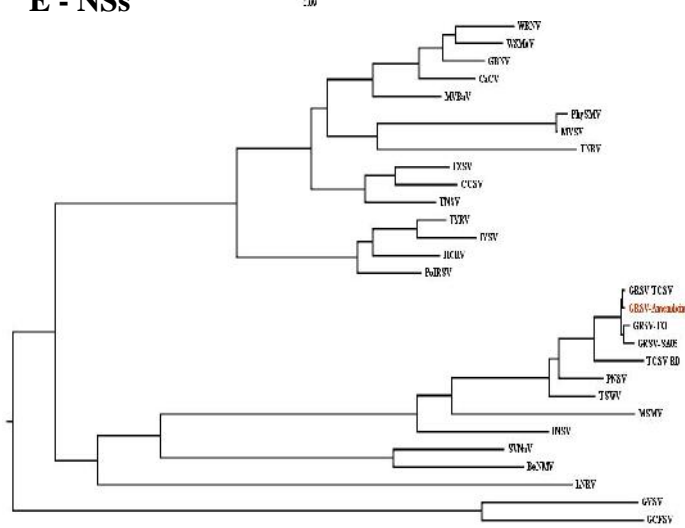
**C - NSm**



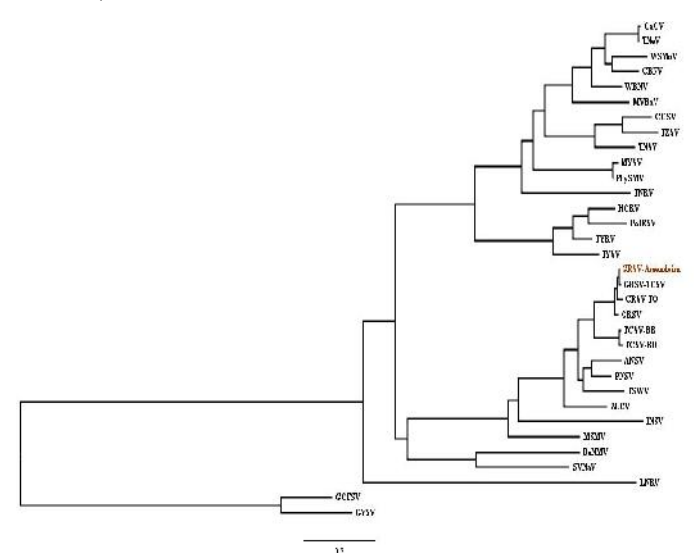
**D - GP**



**E - NSs**



**F - N**

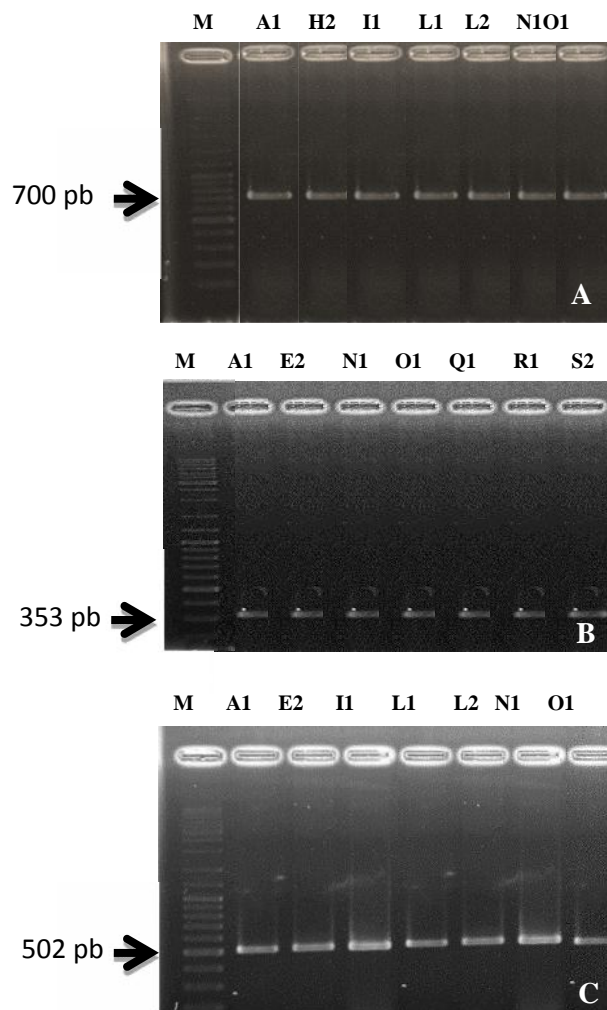


**Fig 4. Schematic representation of the genome organization and phylogenetic position of *Groundnut ringspot virus* -GRSV (genus *Tospovirus*) isolated from peanut (*Arachis hypogaea*) in Brazil (detached in red).** (A) Genomic organization of GRSV, demonstrating tripartite genome, represented by segment L encodes the RNA dependent RNA polymerase (RdRp), segment M encodes a precursor protein of glycoproteins Gn / Gc and NSm cell viral movement protein cell to cell via plasmodesma and segment S encodes the nucleocapsid protein (N) protein with suppressor function of the gene silencing (NSs), (B) Phylogenetic tree using RNA polymerase nucleotide sequences dependent RNA polymerase (RdRp), (C) phylogenetic tree using nucleotide sequences of viral movement protein (NSm). (D) Phylogenetic tree using nucleotide sequences of the glycoproteins (GP). (E) phylogenetic tree using suppressor protein nucleotide sequences of the gene silencing (NSs) and (F) phylogenetic tree using nucleocapsid protein (N) sequences of nucleotides. The trees were constructed by the Neighbour-Joining method, model Tamura-Nei with 1000 bootstrap replicates in Geneious 7.1 program.

**Analysis of *Groundnut ringspot virus* - GRSV isolates using primers for segments L, M and S designed from sequences obtained by *Next-Generation Sequencing* – NGS**

Among 33 samples tested by RT-PCR, 11 were positive to GRSV, varying the genome segments as following: for L, M and S segments - A1 (Santa Adélia), N1 and O1 (Tupã); for L segment - H2 (Itápolis), I1 (Pindorama), L1 and L2 (Tupã); for M segment - E2 (Jaboticabal), Q1, R1 (Guainbê) and S2 (Guarantã) and for S segment - E2, H2, I1 and L2. Specific primers for virus detection assays were designed and synthesized.

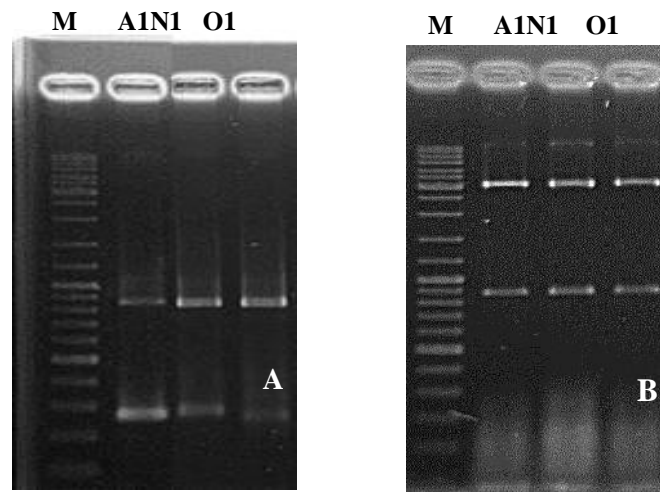
With the primers used for the segment L, the polymerase fragment of approximately 700 bp was obtained. For the segment M, 353 bp of NSm protein region and a 502 bp segment S of NSs protein region were confirmed by RT-PCR (Fig 5). The Sanger sequencing confirmed the presence of GRSV in the samples A1, N1 and O1.



**Fig 5. Result of RT-PCR using specific primers for the segments L, M and S *Groundnut ringspot virus* - GRSV.** (A) Agarose gel at 1% of PCR performed using the primer for segment L showing an amplicon of 700 base pairs (bp). Positive samples for the segment L **A1** (Santa Adélia), **H2** (Itápolis), **I1** (Pindorama), **L1**, **L2**, **N1** and **O1** (Tupã), (B) Amplicons of 353 base pairs generated by the primer for the segment M. Positive samples for the M segment: **A1** (Santa Adélia), **E2** (Jaboticabal), **N1**, **O1** (Tupã), **Q1**, **R1** (Guainbê) and **S2** (Guarantã) and, (C) Amplicons of 502 base pairs generated by primer to the segment samples positive for S. segment S **A1** (Santa Adélia), **E2** (Jaboticabal), **I1** (Pindorama), **L1**, **L2**, and **N1O1** (Tupã). M: Marker 1 kb (Thermo Scientific).

### **Amplification, cloning and sequencing the *Groundnut ringspot virus* - GRSV N gene**

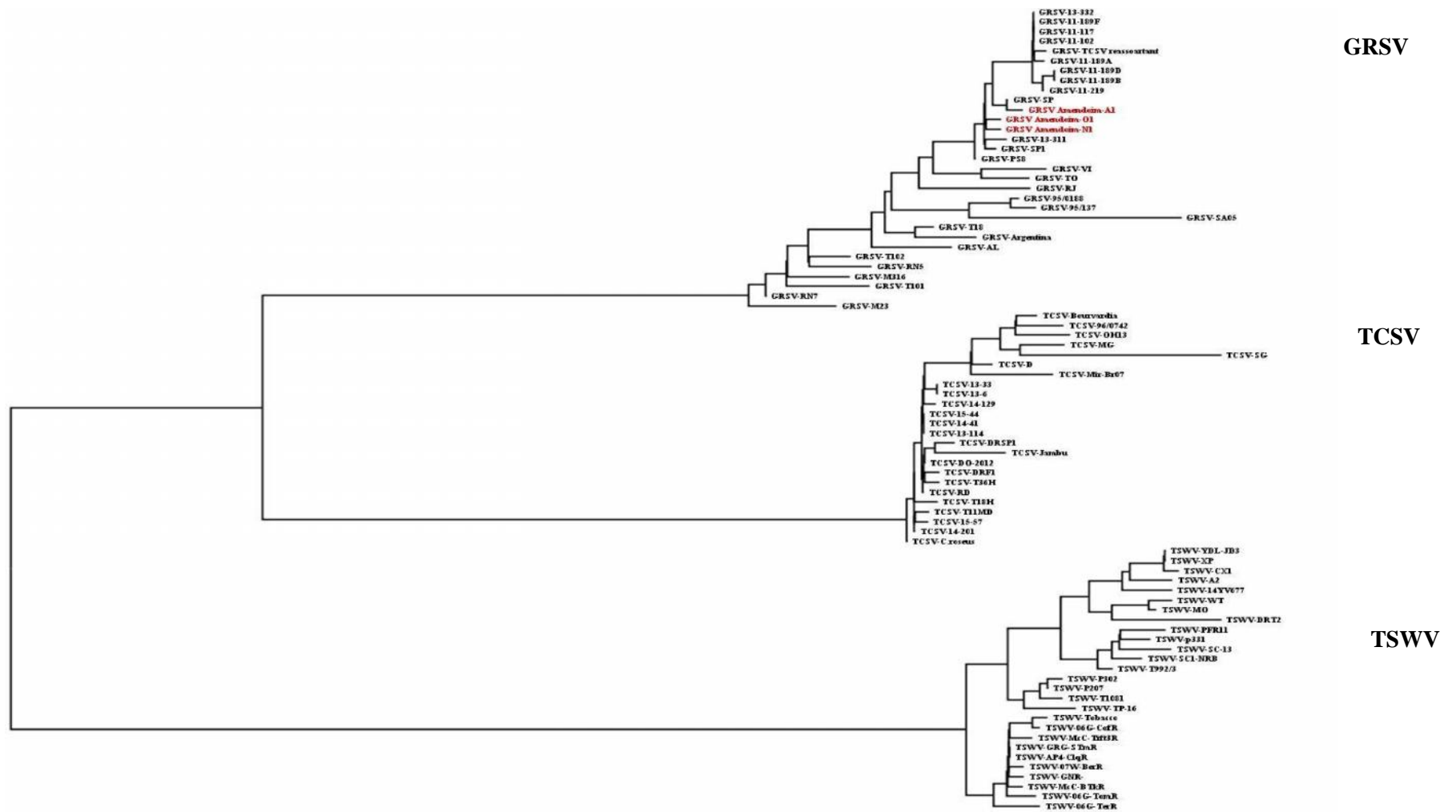
A 900 bp amplicon was obtained by RT-PCR from samples A1 (Santa Adélia), N1 and O1 (Tupã) (Fig 6). The fragments were inserted into pGEM®-T Easy vector (3000 bp). Several white colonies were obtained as result of electroporation. From the positive colonies, one was individually isolated, from which the plasmid DNA was digested with EcoRI restriction enzyme. After digestion, could be noted that there was a release of fragment of approximately 900 bp corresponding to the cloned DNA (Fig 6).



**Fig 6. Results of RT-PCR and cloning of the N protein of *Groundnut ringspot virus* - GRSV.** (A) Agarose gel at 1% of RT-PCR performed using specific primer for amplification of the N gene (segment S), showing an amplicon of 900 base pairs (bp). **M:** Marker 1kb Thermo Scientific, samples **A1** (Santa Adélia), **N1** (Tupã) and **O1** (Tupã) and (B) Cloning confirmation. Agarose 1% gel digestion with EcoRI restriction endonuclease clones N gene (segment S) of GRSV virus originating from the samples A1, N1 O1 and peanut. It is observable the release of approximately 900 bp cloned fragment and the vector pGEM®-T.Easy 3.000 bp.

### **Phylogenetic tree with N gene sequences of *Groundnut ringspot virus* - GRSV, *Tomato chlorotic spot virus* - TCSV and *Tomato spotted wilt virus* - TSWV**

The phylogenetic analysis with the nucleotide sequences of N protein from GRSV, TCSV and TSWV isolates demonstrated that the Brazilian isolate of GRSV from peanut studied in this work grouped in a clade with other GRSV isolated from Brazil, being somewhat distant from recombinant isolate [35] as can be seen in Fig 7. More information tables 5, 6 and 7.



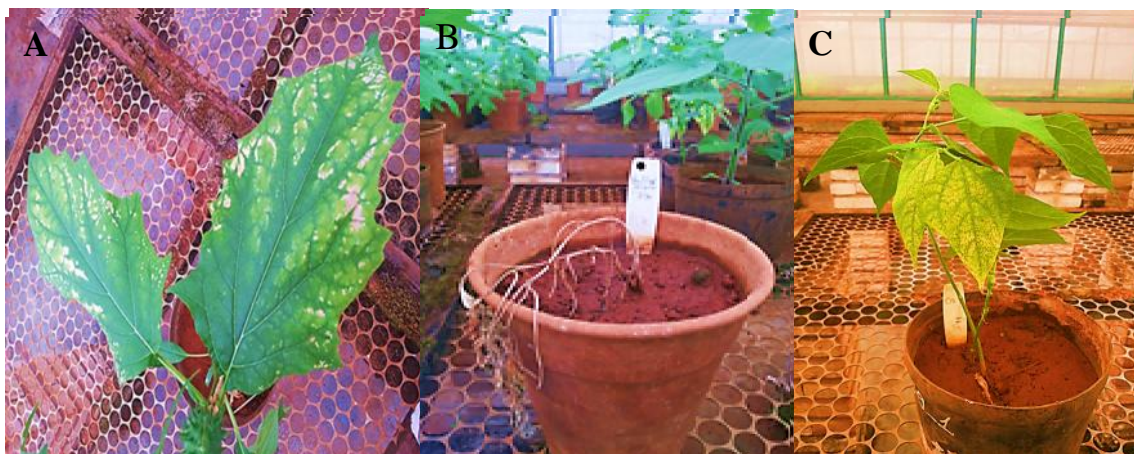
**Fig 7.** Phylogenetic tree of *Groundnut ringspot virus* - GRSV, *Tomato chlorotic spot virus* - TCSV and *Tomato spotted wilt virus* - TSWV isolates. Sequences of nucleotides N protein were used to construct the tree, showing the phylogenetic position of the isolates peanut GRSV A1 (Santa Adélia), N1 and O1 (Tupã) highlighted in red. The trees were constructed by the Neighbour-Joining method, model Tamura-Nei with 1000 bootstrap replicates in Geneious 7.1 program.



### Characterization of virus isolates by host range and Dot-ELISA

The isolates tested A1, N1 and O1 caused symptomatic response in at least one of the indicator species used in this work. Symptoms were observed between 15 and 30 days after inoculation. All three isolates induced necrotic and chlorotic lesions in *D. stramonium* (A1 Fig 8-A), which is a good indicator for GRSV. A1 also caused necrotic lesion in *C. quinoa*. N1 produced chlorosis in *N. tabacum*-TNN and, in *N. benthamiana*, the formation of local necrotic lesions and systemic necrosis followed by plant death (Fig 8-B). The isolate O1 caused yellow spots on inoculated leaves in *P. vulgaris* (Fig 8-C). Most of these symptoms coincided with symptoms described in the literature as typical to GRSV on these plants [46, 47].

In the serological test done with samples from different indicator species inoculated with the 3 peanut samples, showed positive reaction to GRSV.



**Fig 8. Plants inoculated with three virus isolates from peanut (*Arachis hypogaea*) collected in different locations (in parentheses) of São Paulo state showing characteristic symptoms of virus. (A) Local necrotic lesions and chlorotic *Datura stramonium* (sample A1- Santa Adélia), (B) Formation of necrotic local lesions and systemic necrosis followed by plant death in *Nicotiana benthamiana* (sample N1- Tupã), (C) yellow spots on inoculated leaves in *Phaseolus vulgaris* (sample O1-Tupã).**

### Discussion

Peanut is an important leguminous crop and Brazil occupies the 17<sup>th</sup> position in world production, with the state of São Paulo comprising 91.4% of the national output [3]. In 2012/13, 2013/14 and 2014/15 peanut-growing seasons, high incidence of peanut plants



showing typical symptoms of tospoviruses in several counties of the state of São Paulo were observed.

Many types of tests can be used for detecting of virus species. Nowadays, the metagenomics coupled with high-performance sequencing (*Next-Generation Sequencing* - NGS) has allowed not only detection, but also the elucidation and exploitation of the diversity of microorganisms in environmental samples included viruses. Any total nucleic acid extracted from plant tissue infected with virus can be used for identification by NGS. The existing viral metagenomic in biological or environmental samples is called "virome" [41].

As an example may be mentioned a new *Cucumovirus* detected in *Liatrix spicata* (L.) Willd., which was provisionally called Gayfeather mild mottle virus [48]. In sweet potato (*Ipomoea batatas* (L.) Lam.) were identified two species of dsDNA virus (genus *Badnavirus*) and one species of ssDNA virus (genus *Mastrevirus*) were identified [49]. In pepper (*C. annuum* L.) and eggplant Dombrovsky et al. [42] detected the complete genome sequence of two new viruses called *Pepper yellow leaf curl virus* (genus *Polerovirus*) and *Eggplant mild leaf mottle virus* (genus *Ipomovirus*). In tomato, the sequence of complete genome of a new species of *Potyvirus* named *Tomato necrotic stunt virus* was determined [37]. In addition, four new viral species, classified in genus *Potyvirus*, *Sadwavivirus* and *Trichovirus*, were found in 17 species of wild plants in Australia [40, 41].

For peanut crop, however, the present work using a metagenomic analysis did not show a great viral diversity using the Illumina MiSeq platform. It was possible the detection and recovery the complete genome sequences of PeMoV (genus *Potyvirus*) GRSV (genus *Tospovirus*) from peanut samples collected in producing areas of 10 counties of the state of São Paulo.

The PeMoV is a *Potyviridae* family member and its transmission occurs in a non-circulative, not persistent manner by aphids, mechanically and peanut seeds. The PeMoV species has been detected worldwide, and isolates from countries such as Ivory Coast, the United States, Sudan and many others, have been detected and characterized by using serological and biological methods [49, 50, 51].

Recently, the first report of PeMoV in the forage peanut *A. glabrata* Benth species in North America, using specific primers with sequences of coat protein was published [52]. Spiegel et al. [53] performed the molecular characterization of a PeMoV isolate from peanut

by RT-PCR and the phylogenetic analysis indicated that it is closely related with a PeMoV isolate from Australia. In Iran the PeMoV from peanut was detected using biological, serological and molecular tests [19].

In Brazil, the PeMoV was firstly reported by Kitajima [15] that named "peanut mosaic". Lately, it was detected by Andrade et al. [16] infecting peanut in São Paulo state and by Nascimento et al. [17] in Paraíba state. This virus was also found in forage peanut (*A. pintoii* Krapov.) [54] and soybean [55]. In all these works the detection was performed by serological tests, inoculation in range indicator host and electron microscopy.

The CP amino acid sequences identity smaller than 80%, nucleotide sequence identity less than 76% and differences in the cleavage site of the polyprotein are the main criteria for demarcation species within the *Potyviridae* family [13]. Phylogenetic analysis and tree construction were done using the result of metagenomic and two other PeMoV-CP sequences deposited in GenBank. The first was obtained from *Cassia* sp. (United States) [20] and the other from soybean (South Korea) [21]. In both cases, the detection was performed using specific primers for the CP and the obtained amplicons were cloned and sequenced. The genome of the PeMoV isolate reported here revealed 98% and 97% identity to the South Korea (PeMoV-Habin) and United State (PeMoV-Strain M) isolates, respectively (Fig 3). Taking it into consideration, this work is pioneer in the detection of complete genome sequence of PeMoV from peanut by metagenomic using NGS.

Several species of tospovirus have been identified around the world on the basis of host range, transmission specificity by different thrips species and genetic analysis of the N protein [56, 57, 58, 59, 25, 19, 32, 30, 29, 31, 58, 33, 34].

The species classified in the genus *Tospovirus* (*Bunyaviridae* family) have a wide geographical distribution [60]. They can be found throughout the world and cause disease in a wide range of cultivated and wild plants as onion, tomato, tobacco, cubiu, pepper, cucumber, lettuce, zucchini (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus* L.), melon (*Cucumis melo* L.), potato (*Solanum tuberosum* L.), peanuts, soybeans, beans, mulberry (*Morus* sp.), and ornamental plants as black bindweed (*Polygonum convolvulus* L.), lily-spider (*Hymenocallis littoralis* L.), glass-of-milk (*Zantedeschia aethiopica* L.), iris and chrysanthemum [61, 40, 6, 63, 64, 30, 65, 32, 31, 66, 67, 34]. Viruses of this genus are

transmitted by insects of the order Thysanoptera commonly known as thrips (Family: *Thripidae*) in a relationship characterized as propagative circulative [28].

The taxonomic criteria for determining a new species in the genus *Tospovirus* are based on differences in host range, symptoms, and transmission test with thrips, serological tests and comparative analysis of the identity of sequences of nucleocapsid (N) protein. When the N protein sequence identity of a tospovirus isolate is less than 90%, compared with all known sequences, it can be considered a new species [28, 57]. However, several studies show that the amino acid sequences of NSm protein, glycoprotein Gn and Gc and L protein have similar phylogenetic behavior the N protein, suggesting that they may all reflect the natural evolution of tospovirus species [68, 69, 70].

In Brazil there is a great diversity of tospoviruses with the presence of isolates of TSWV, GRSV, TCSV, ZLCV, CSNV, IYSV and BeNMV, among others, have been identified in various crops throughout the country. Phylogenetic analyses have shown a clade that represents the group of American viruses and a clade that represents the Eurasian group. However, this division of clades do not correspond to the geographical distribution of tospoviruses TSWV, INSV, IYSV, among others, which are distributed worldwide probably because of the wide distribution of their delivery and / or exchange of infected plant material (Fig 4) [64].

The GRSV was first described in peanut in South Africa [27]. After that, many reports have been done in the same and other crops in different countries: tobacco from Brazil [61], peanuts and soybean from Argentina [64]; tomato, pepper, tomatillo (*Physalis philadelphica* L.) and eggplant (*Solanum melongena* L.) from South Florida [71, 35] and peanut from Brazil [46].

In a general way for viruses the major sources of variability are the mutation, recombination and pseudo-recombination [72]. Population analysis of several DNA and RNA viruses show that recombination may be the largest source of variation to occur evolution [73, 74]. The multipartite genomes as those of tospoviruses allow recombination events. There may be a rearrangement among isolates of same virus species as observed for TSWV or different species as observed in GRSV and TCSV [35].

The GRSV genome detected and recovered in this metagenomic study showed three segments. The first them, L segment encoding the RNA dependent RNA polymerase (RdRp).

The M segment encoding the glycoproteins (Gn/Gc) and viral movement protein (NSm). A lower segment S encoding the protein of the nucleocapsid (N) and protein with suppressor function of the gene silencing (NSs) (Fig 4).

Phylogenetic analyzes based on the results of metagenomics for segment L (RdRp) and segment S (N and NSs), revealed that the peanut isolated GRSV grouped with isolated GRSV, getting closer phylogenetically recombinant isolated GRSV and TCSV [35] and GRSV isolated watermelon (unpublished data) showing 98% and 97% identity with the isolates, respectively (Fig 4). For the M segment, the result is different, since the GRSV isolate from peanut pooled with the recombinant isolate and isolates TCSV virus Dominican Republic and Brazil, revealing a low genetic diversity of these isolates, indicating that probably these species are sharing the same segment (Fig 4). Webster et al. [35] reported in Florida through the genome sequence comparisons, a rearrangement that occurred between GRSV and TCSV wherein L and S are segments of GRSV and the M segment of TCSV (eg:  $L_{\text{GRSV}} - M_{\text{TCSV}} - S_{\text{GRSV}}$ ). All GRSV isolates characterized in Florida have a genome in which L and S segments of GRSV and the segment M of TCSV. Unlike TCSV isolates reported in Florida, those from Puerto Rico and the Dominican Republic have all segments of TCSV species, not showing recombination between species [75, 76].

Phylogenetic trees constructed from sequences obtained using primers synthesized from metagenomic sequences revealed that for the segments L, and S (NSs region) whose amplicons of 700 and 502 bp, respectively (data not shown), the results were the same compared to those that were used to construct phylogenetic trees illustrated in Fig 4. It was observed that isolates GRSV A1, N1 and O1 is positioned closest phylogenetically to recombinant isolate of GRSV and TCSV and a GRSV isolate from watermelon. As for the segment M (region NSm), amplicon of 353 bp, peanut isolates gathered between them demonstrating a lower genetic diversity, getting closer phylogenetically of GRSV isolate from watermelon (unpublished data, personal information) and a GRSV isolate from South Africa [27].

With specific primers synthesized for GRSV it was possible viral detection in 11 out of 33 samples collected and analyzed. This result may be related to the kind of previous crop in the county in which the plant was collected. Some negative samples from the places like Rancharia and Marília were observed were pastures have been planted, prior to peanuts. In this context, it is believed that crop rotation with pasture may interfere reducing the virus

occurrence in the field by reducing viral inoculum. This indicates the possibility of using this strategy for tospovirus control by cultivation plant species not host of the virus and/or vector.

It was also observed that the percentage of positive samples in the counties of Santa Adélia, Jaboticabal, Itápolis, Pindorama, Tupã, Guainê and Guarantã was low. These observations support the hypothesis above. In these locations, sugarcane was planted previously to peanut, which is indicated for use in crop rotation not to be host of thrips. Another issue to be taken into consideration is the distance between producer's counties of peanuts. As an example, the county of Pindorama and Santa Adélia that are in the same administrative region of São José do Rio Preto and near to Itápolis and Jaboticabal lying in other administrative regions. On the other hand, among the evaluated cities, Rancharia and Marília are a little more distant from the cities that had at least one positive sample in this study.

Another important factor to be considered refers to the natural reservoir and source of viral inoculum present in alternative and weeds, which have not been evaluated here. In a survey of *Frankliniella schultzei* hosts, realized at UNESP Campus Jaboticabal, state of São Paulo, out of 43 species of weeds analyzed, 19 were host of this insect, that was also observed on plants of radish (*Raphanus sativus* L.), turnip (*R. raphanistrum* L.) and mustard (*Sinapis arvensis* L.) with 45, 27 and 17% respectively [77].

Phylogenetic analyzes of the N protein nucleotide sequences, widely used for separation of species in this genus, showed that the peanut isolate of GRSV reported this work grouped with other GRSV isolates from Brazil, most of them also got from peanut (Fig 7). Similar results were obtained in a study conducted with 14 GRSV peanut isolates in Córdoba – Argentina. The phylogenetic analysis based on the nucleotide sequence of the N protein showed segregation of GRSV isolates into three groups. The Argentinian GRSV isolates grouped together, while the GRSV isolates from South Africa and Brazil were in separate clades, indicating that the N gene protein is highly conserved and that the degree of relatedness between GRSV isolates are more closely related with the geographical origin [78]. This information are in agreement with previous studies on tospoviruses [52, 79].

The biological and serological results corroborate the results of metagenomics, obtained here, demonstrating the presence of GRSV in peanut producing counties of São

Paulo. Similar data were obtained by Camelo-garcía et al. [47], studying high incidence of peanut plants with symptoms and GRSV detection in Itápolis.

This work reports the first complete genome sequence of PeMoV infecting peanut. It was also possible to identify and recover the complete sequence of GRSV in peanut producing areas in the State of São Paulo. Since the GRSV isolated from peanut shown to be closely related phylogenetically to a recombinant isolate ( $L_{GRSV}$ ,  $M_{TCSV}$ ,  $S_{GRSV}$ ), for all segments, it is possible to have GRSV recombinants in peanut in Brazil. This is believed by the fact, that GRSV and TCSV species have a low genetic diversity among themselves (segment M: NSm and Gn/Gc) suggesting that they may be sharing this segment. It was also noted by the phylogenetic analyzes that N protein of the GRSV isolates analyzed in this study (A1, N1 and O1) positioned in a clade with GRSV isolates from Brazil previously described.

### **Acknowledgments**

We thank Dr. Ignacio Godoy and João Santos of Instituto Agronômico de Campinas-SP the aid and essential help in collections of peanut samples. In addition, the Laboratory of Virology Plant at the Universidade de Brasilia for the support and infrastructure available to carry out the work.

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**CONCLUSÕES GERAIS**



## CONCLUSÕES GERAIS

- A detecção e recuperação do genoma completo do *Peanut mottle virus*-PeMoV (gênero *Potyvirus*) e *Groundnut ringspot virus* - GRSV (gênero *Tospovirus*), conseguidas neste trabalho, foio primeiro caso registradoem nível mundial, por meio do uso de tecnologia de sequenciamento de alto desempenho e análise metagenômica, a partir de plantas de amendoim;
- Pelas análises filogenéticas para os três segmentos genômicos de tospovírus com a proteína N, verificou-se que o isolado de GRSV estudado neste trabalho se agrupou com isolados obtidos de amendoim e melancia e um isolado recombinante de GRSV e TCSV de tomate.
- Com base nas análises filogenéticas para as proteínas codificadas por todos os segmentos de GRSV, acredita-se que as espécies de GRSV e TCSV compartilhem do mesmo segmento M.
- Pela análise filogenética das sequências disponíveis da capa proteica de isolados do PeMoV, conclui-se que o isolado brasileiro de amendoim, estudado neste trabalho, é filogeneticamente relacionado com isolados obtidos de soja e de *Cassia* sp., com identidade de 98 e 97%, respectivamente.