Ciência Rural, Santa Maria, v.51:4, e20200702, 2021
http://doi.org/10.1590/0103-8478cr20200702
ISSNe 1678-4596
CROP PROTECTION

Near-complete genome sequence and seed transmission evaluation of *Physalis rugose mosaic virus* from southern Brazil

Amanda Savi[1] Eduardo Silva Gorayeb[1] Samara Campos Nascimento[1] Caroline Bolson de Faria[1] Thor Vinícius Martins Fajardo[2] Antônio Nhani Junior[3] Amauri Bogo[1] Ricardo Trezzi Casa[1] Fabio Nascimento da Silva[1*]

[1]Programa de Pós-Graduação em Produção Vegetal, Universidade do Estado de Santa Catarina (UDESC), 88520-000, Lages, SC, Brasil. E-mail: fabio.silva@udesc.br. *Corresponding author.
[2]Embrapa Uva e Vinho, Bento Gonçalves, RS, Brasil.
[3]Embrapa Informática Agropecuária, Campinas, SP, Brasil.

ABSTRACT: *Physalis rugose mosaic virus (PhyRMV) causes severe damage to Physalis peruviana L., affecting vegetative parameters, fruit quantity and quality. The aim of this study was to perform a molecular characterization of PhyRMV associated with P. peruviana from commercial fields in the municipality of Lages, Santa Catarina State, Southern Brazil, and to evaluate its transmission by seeds. Plants displaying mosaic, dwarfism, and leaf malformation symptoms were collected from P. peruviana. Double-stranded RNA was extracted and submitted to cDNA library synthesis and high-throughput sequencing (HTS). For the virus transmission assay, seeds from PhyRMV-infected plants were used, and viral infection in seedlings was verified using symptomatic and molecular diagnosis. PhyRMV RNA has 4162 nucleotides (nts) and a genomic organization similar to that of other sobemoviruses and shares 97% nt identity with the previously characterized PhyRMV Piracicaba isolate. Results indicated the unlikely transmission of PhyRMV by physalis seeds.*
Key words: *characterization, PhyRMV, Sobemovirus.*

Sequência do genoma completo e avaliação da transmissão por sementes do *Physalis rugose mosaic virus* no sul do Brasil

RESUMO: *Physalis rugose mosaic virus (PhyRMV) causa danos severos em Physalis peruviana L., afetando características vegetativas, a quantidade e qualidade de frutos. Os objetivos desse estudo consistem na caracterização molecular do PhyRMV associado a P. peruviana coletada em campos de produção em Lages, Santa Catarina, Brasil; e avaliar a transmissão do vírus por sementes. Plantas apresentando sintomas de mosaico, deformação e nanismo foram coletadas de P. peruviana. Amostras foliares dessas plantas foram utilizadas para extração de RNA de fita dupla, síntese de biblioteca de cDNA e sequenciamento de alto rendimento. No experimento de transmissão, sementes obtidas de plantas infectadas por PhyRMV foram utilizadas, e a infecção viral nas plântulas foi avaliada por inspeção visual de sintomas e diagnóstico molecular. O RNA viral apresentou 4162 nucleotideos (nts), a organização genômica foi similar à de outros sobemovírus e apresentou 97% de identidade de nucleotideos com o isolado de Piracicaba de PhyRMV previamente caracterizado. Os resultados obtidos sugerem que é improvável a transmissão de PhyRMV por sementes de physalis.*
Palavras-chave: *caracterização, PhyRMV, Sobemovirus.*

Physalis peruviana L. is a small fruit belonging to the Solanaceae family, well known for its high nutritional and economic value. In the American continent, the cultivation of plants of this solanaceous species has been increasing in recent years, especially in higher altitude regions in tropical and subtropical countries (FISCHER; MIRANDA, 2012). *P. peruviana* can be asexually propagated, but the main propagation form is through seeds (MUNIZ et al., 2014).

Twenty-one viruses belonging to 14 genera have been reported to infect *P. peruviana* worldwide (AGUIRRE-RÁQUIRA et al., 2014; DALLOS et al., 2010; FARIÑA et al., 2019; GÁMEZ-JIMÉNEZ et al., 2009; GARCÍA et al, 2020; GRAÇA et al. 1985; GUTIÉRREZ et al., 2015; KISTEN et al., 2016; PRAKASH et al., 1988; SALAMON; PALKOVICS, 2005; THOMAS; HASSAN, 2002; TRENADO et al., 2007). In Brazil, there have been reports of two orthotospoviruses, tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) (EIRAS et al., 2012; ESQUIVEL et al., 2018), and a putative new sobemovirus named physalis rugose mosaic virus (PhyRMV) (FARIÑA et al., 2019).

Received 07.27.20 Approved 10.13.20 Returned by the author 11.18.20
CR-2020-0702.R1

Plants of *P. peruviana* infected with PhyRMV showed symptoms of mosaic, leaf malformation, and dwarfism (Fariña et al. 2019 and Figure 1A). This virus has already been detected in plants from three Brazilian States (Paraná, Santa Catarina and São Paulo), causing severe symptoms and reduced production (FARIÑA et al., 2019; GORAYEB et al., 2020). Additionally, it has the potential to infect other important species such as *Capsicum annuum*, *Nicotiana tabacum*, and *Solanum lycopersicum* (FARIÑA et al., 2019).

Transmission of viruses through seeds is an intrinsic property of about 25% of viruses that infect plants (JOHANSEN et al., 1994; SASTRY, 2013). Among sobemoviruses, southern bean mosaic virus (SBMV), southern cowpea mosaic virus (SCPMV), sowbane mosaic virus (SoMV), subterranean clover mottle virus (SCMoV), and snake melon asteroid mosaic virus (SMAMV) are seed-transmissible (SÕMERA et al., 2015). Seed transmission has epidemiological importance, since a pathogen transmitted by seeds has considerable potential for survival and longevity and can escape unfavorable conditions (SALAUDEEN, 2012). In addition, the ability to infect a seed may favor virus spread to new areas, as is the case of the SCMoV, which is believed to have been introduced in Australia through infected seeds during European colonization (JONES, 2004).

Given the emerging character of PhyRMV and its potential to inflict damage on *P. peruviana* and other important hosts, it is essential to characterize the genome of a greater number of isolates and to investigate its modes of transmission. Therefore, the aim of this study was to perform a molecular characterization of PhyRMV associated with *P. peruviana* from a commercial field in the municipality of Lages, Santa Catarina State, Southern Brazil and to evaluate the seed transmission of PhyRMV.

Healthy *P. peruviana* seedlings were transplanted into 30 vessels (25 liters), each containing a mixture of substrate and soil at a 3:1 ratio, and kept in a greenhouse at 24 °C (±2 °C). Inoculation via buffered plant extract [0.02M sodium phosphate buffer (pH 7.0) plus 0.02M sodium sulfite] occurred 20 days after transplantation. The plants were divided into two treatments: (*i*) 10 plants inoculated only with buffer and (*ii*) 20 plants inoculated with PhyRMV. The viral isolate used in this study was collected from *P. peruviana* in a commercial field in the municipality of Lages (27°48'57"S; 50°19'33"W), and the isolate was kept in *P. peruviana* in a greenhouse at 24 °C (±2 °C).

For molecular detection of PhyRMV in inoculated plants, 100 mg of symptomatic *P. peruviana* leaves were homogenized in liquid nitrogen and submitted to total RNA extraction using TRi reagent (Sigma Aldrich), according to the manufacturer's instructions. For the synthesis of complementary DNA (cDNA), the enzyme RT-MMLV (200 U/µL) (Promega) was used according to the manufacturer's instructions. PCR was performed using the enzyme goTaq Flexi DNA polymerase (Promega) according to the manufacturer's instructions, with the specific primers for PhyRMV, Sobemo 1F (5'-TAG CCA AGC TCA ATC CAT TT-3') and Sobemo 1R (5'-GTC TTA GGC CAA GAA GTC AA-3') (FARIÑA et al., 2019), with 1 min of annealing at 53 ºC. The PCR products were separated by 1% agarose gel electrophoresis, stained with GelRed (Biotium), visualized under UV light, and photographed.

Double-stranded RNA (dsRNA) extraction was performed from a set of six PhyRMV-infected plants showing typical symptoms of viral infection using 15 g of leaf tissue, following the protocol described by Valverde et al. (1990) with minor modifications (VALENTE et al., 2019). The dsRNA samples were placed in RNA Stable tubes (Biomatrica) and dried in a Speed Vac (Eppendorf Concentrator Plus) for 1.5 h; the dsRNAs were subjected to quality control and high-throughput sequencing (HTS) performed at Proteimax Biotecnologia Ltda (São Paulo, SP). HTS readings were generated from sequencing libraries generated using TruSeq stranded total RNA with Ribo-zero Plant and the Illumina HiSeq 4000/NovaSeq platform. The quality of the cDNA library was verified with the software FastQC, and the low-quality adapters and readings were removed by Trimmomatic (BOLGER; et al., 2014). The data obtained by HTS were analyzed using the software SPAdes v.3.11.1 (BANKEVICH et al., 2012) for contig assembly. The resulting contigs were analyzed for similarity to public databases, and sequences related to complete viral genomes were identified.

HTS validation was performed using 7 µg of purified dsRNA, which was denatured at 94 °C for 5 minutes followed by synthesis of the cDNA strand using the oligo dT primer and the ImProm II Reverse Transcription System kit, as recommended by the manufacturer. Then, the PCR reaction was performed as previously described. The amplified fragments in the PCR reactions were separated by 1% agarose gel electrophoresis, stained with GelRed (Biotium), visualized under UV light in a transilluminator, and photographed. DNA fragments of the expected size were sent for sequencing (ACTGene Molecular analysis), using the Sobemo 1F and Sobemo 1R primers.

ORF prediction of the contigs was performed using the ORF Finder program in NCBI

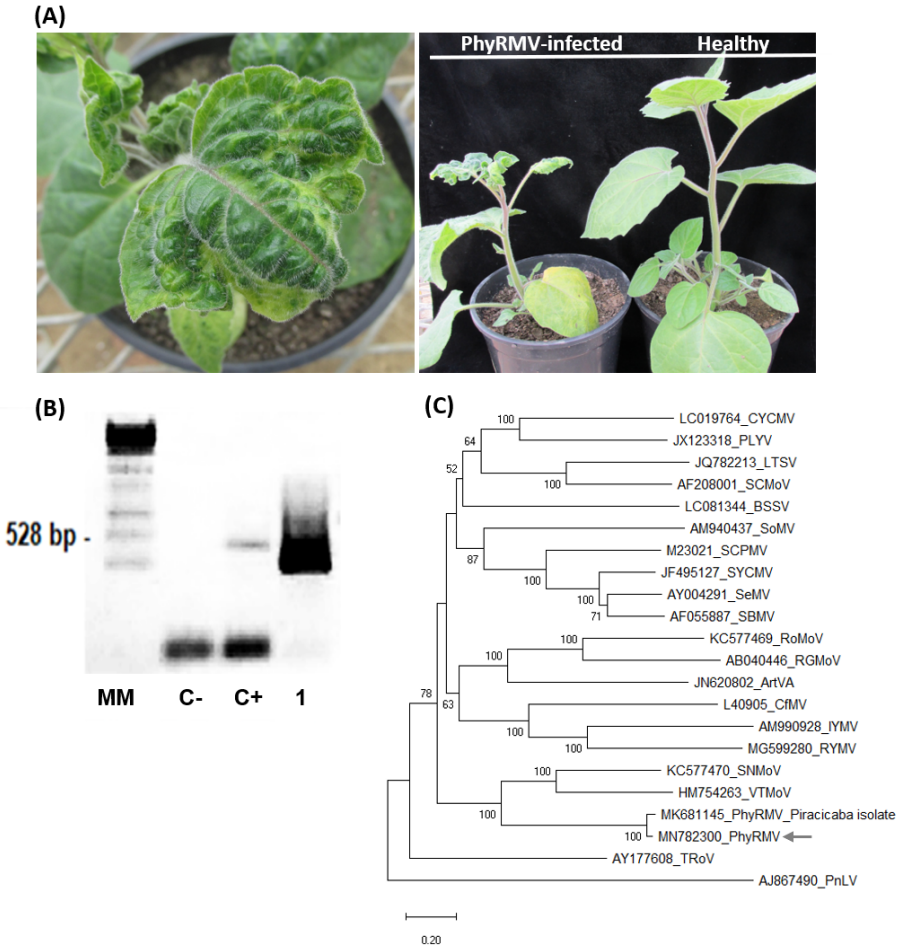

Figure 1 - (A) *Physalis peruviana* infected by physalis rugose mosaic virus (PhyRMV) showing mosaic, malformation and blister symptoms (left), and dwarfism (right). (B) Electrophoretic analyses of the PCR products for the detection of PhyRMV from original samples collected. MM: 1 kb molecular ladder; C-: not-infected *P. peruviana* (negative control); C+: PhyRMV-infected *P. peruviana* (positive control); 1: original dsRNA extracted from *P. peruviana* leaves. (C) Phylogenetic relationship based on the alignment of the PhyRMV near-complete genome and other members of the genus *Sobemovirus*. General Time Reversible (GTR) model with gamma distribution (G) was used and bootstrap support of 8,000 replications. The alignment for phylogeny was performed with the MUSCLE tool available in the MEGA X program. The numbers on the branches indicate the bootstrap values. Members of the *Sobemovirus* genus are: artemisia virus A (ArtVA; JN620802), blueberry shoestring virus (BSSV; LC081344), cocksfoot mottle virus (CfMV; L40905), cymbidium chlorotic mosaic virus (CyCMV; LC019764), imperata virus (IYMV; AM990928), lucerne transient streak virus (LTSV; JQ782213), papaya lethal yellowing virus (PLYV; JX123318), rice yellow mottle virus (RYMV; MG599280), rottboellia yellow mottle virus (RoMoV; KC577469), ryegrass mottle virus (RGMoV; AB040446), sesbania mosaic virus (SeMV; AY004291), solanum nodiflorum mottle virus (SNMoV; KC577470), southern bean mosaic virus (SBMV; AF055887), southern cowpea mosaic virus (SCPMV; M23021), sowbane mosaic virus (SoMV; AM940437), soybean yellow common mosaic virus (SYCMV; JF495127), subterranean clover mottle virus (SCMoV; AF208001), turnip rosette virus (TRoV; AY177608), velvet tobacco mottle virus (VTMoV; HM754263), and physalis rugose mosaic virus (PhyRMV; MK681145). The arrow indicates the isolate characterized in this study.

(http://www.ncbi.nlm.nih.gov/projects/gorf/); identification of conserved and functional domains of proteins was performed using the SMART tool (http: //smart.embl-heidelberg.de/) (LETUNIC et al., 2015). The phylogenetic tree was built using the maximum likelihood method implemented in the MEGA X program (KUMAR et al., 2018). In the phylogenetic analyses, a member of the genus *Polemovirus* [poinsettia latent virus (PnLV)] was used as outgroup. *Polemovirus* and *Sobemovirus* belong to the family *Solemoviridae*, Order *Sobelivirales*, Class *Pisoniviricetes*, Phylum *Pisuviricota*, Kingdom *Orthornavirae*, Realm *Riboviria*.

The nucleotide (nt) and amino acid (aa) identities of the complete genomes and coding regions of the isolate characterized in this study were compared with those of the PhyRMV isolate Piracicaba, using the MUSCLE and Clustal Omega algorithms for nt and aa, respectively (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

For analysis of seed transmission, the presence of the virus in seeds and seedlings was evaluated. To identify virus presence in any part of the seed, six PhyRMV-infected *P. peruviana* plants supplied six seed samples, each containing 70 mg (~70 seeds) of *P. peruviana*. Each seed sample was homogenized in liquid nitrogen, and the total RNA was extracted, using Tri Reagent (Sigma Aldrich), under manufacturer recommended conditions. After this, the cDNA was synthesized, and a subsequent PCR to detect PhyRMV was performed as previously described.

Subsequently, another experiment was carried out for seed transmission evaluation, in which 300 seeds were sown in trays containing autoclaved substrate, inside an insect-proof cage, to develop at least 200 seedlings (since PhyRMV reduce germination rate) (GORAYEB et al., 2020), that were used in each biological replication (three trials, 600 seedlings in total). After the emergency, the seedlings were checked periodically for the presence of symptoms over 50 days. Subsequently, total RNA was extracted (firstly from pools of 10 plants, followed by a new extraction of each plant separately in the case of a positive reaction), the cDNA was synthesized, and a PCR to detect PhyRMV was performed as previously described.

The original *P. peruviana* collected in the field, plants used to maintain the viral inoculum, and plants inoculated with PhyRMV used in experiments tested positive by RT-PCR using the specific primers Sobemo 1F and Sobemo 1R (data not shown).

Using HTS, a total of 82,710,494 reads were generated from the analyzed sample. The sample showed a good yield, considering the total number of sequenced bases and total number of readings and presented a CG/AT content within the expected range (49.23%). Additionally, the parameters related to quality (Q20) were higher than 97.9%, indicating HTS of excellent quality. After assembly, 29,364 contigs were generated, with 73 contigs homologous to viral sequences. A detailed analysis of these contigs and comparison with already well-characterized viral species confirmed that they contained the near-complete sequence of the PhyRMV genome, isolate PhyRMV:BR:SC:01:2 from the municipality of Lages, Southern Brazil (GenBank accession number MN782300). To confirm the presence of the PhyRMV in the dsRNA used for HTS, the specific primers Sobemo 1F and Sobemo 1R were used, which target a 528-bp fragment of the RdRp gene (Figure 1B), which shares 100% and 97% nt identity with PhyRMV characterized in this study and the Piracicaba isolate of PhyRMV, respectively.

The genomic sequence of the PhyRMV characterized here consists of 4,162 nts with identical genomic organization to that described for the sobemovirus PhyRMV Piracicaba isolate (FARIÑA et al., 2019). The sequence is composed of four open reading frames (ORF1, ORF2a, ORF2b, and ORF3). The 5 'and 3' untranslated regions (UTRs) have 76 and 59 nts, respectively.

ORF1 (nt 77–529; 150 aa) encodes the putative P1 protein, essential to the systemic movement of the virus and suppression of gene silencing (TRUVE; FARGETTE, 2012). ORF2a (nt 548–2317; 589 aa) encodes the putative P2a polyprotein with a typical serine protease motif H(X34)D(X63)TXXGXXGS and the conserved motif WAD, followed by an ED-rich region (TAMM; TRUVE, 2000). After self-processing, it produces viral genome-linked protein (VPg) and the proteins P10 and P8. ORF2b (nt 1750–3561; 603 aa) is translated via −1 ribosomal frameshift from ORF2a (MAKINEN et al., 2000) and produces a protein with an RNA-dependent RNA polymerase domain (RdRp). The RdRp domain contains the conserved motif G(X3)T(X3)N(X19)GDD (KOONIN, 1991). ORF3 (nt 3119–4102; 327 aa) overlaps the 3' region of ORF2b and is translated from a subgenomic RNA (sgRNA), producing the capsid protein. An additional ORF, named ORFx (nt 526–855; 109 aa), overlaps with the 5' region of ORF2a and is possibly initiated by an atypical initiation codon (AUA) reported at nt position 526 (LING et al., 2013).

The PhyRMV isolate characterized in this study is very similar to the previously characterized

PhyRMV Piracicaba isolate (MK681145, 97% nt identity) (FARIÑA et al., 2019). The species demarcation criterion for the genus *Sobemovirus* is genome sequence identity less than about 75% (TRUVE; FARGETTE, 2012), confirming the etiology of the virus characterized in this study. In a comparative analysis of the coding regions of two PhyRMV isolates, the nt and aa identity, respectively, was 96.3% and 97.3% for ORF1, 97.5% and 98.1% for ORF2a, 97.4% and 98.3% for ORF2b, 97% and 98.2% for ORF3, 98.9% and 97.3% for ORFX. Phylogenetic analysis of the near-complete PhyRMV genome and those of other sobemoviruses corroborates the comparative nt and aa data (Figure 1C). The PhyRMV characterized in this study clusters with the PhyRMV Piracicaba isolate and is closely related to solanum nodiflorum mottle virus (SNMoV) and velvet tobacco mottle virus (VTMoV), reported in Australia infecting solanaceous plants (ARTHUR et al., 2010; SÕMERA; TRUVE, 2017) (Figure 1C).

A comparison of the nt sequence of the two PhyRMV isolates revealed 123 changes throughout the genome, the majority of which are considered synonymous substitutions, which do not involve a change in the aa sequence (Figure 2). However, changes in aa were observed at 31 positions (Figure 2), most of them were observed in replication-associated protein (12 aa changes). All coding regions, except for ORFX, presented a greater number of synonymous substitutions (Figure 2), indicating that these regions are under negative selection. Analysis of other viruses shows that in most instances selection is negative (GARCÍA-ARENAL et al., 2001).

When verifying seed transmission of PhyRMV, plants infected with PhyRMV were used to provide seeds for a preliminary trial to detect PhyRMV, in which the virus was detected in one of the six *P. peruviana* seed samples evaluated (data not shown). In transmission analysis, no symptoms were observed in any of the evaluated seedlings (600), and the molecular analyses confirmed the absence of PhyRMV-infection (Table 1), indicating no evidences of transmission by seeds. The presence of the virus in the seed is not a guarantee of transmission to the seedling, and although molecular analyses indicated the presence of the virus in the seeds, the PhyRMV was not able to reach the seedlings. It is worth mentioning that the transmission assay was performed in triplicate, and the results were congruent (Table 1). Transmission by seeds is reported in 23% of sobemoviruses (SÕMERA et al., 2015). Allarangaye et al. (2006) reported for the first time the non-transmission of rice yellow mottle virus (RYMV) through seeds of plants of the wild rice species *Oryiza barthii* and *Oryza longistaminata* and plants of four wild host species (*Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *Eragrostis tenella*, and *Eragrostis tremula*). Together, these results indicated that; although, transmission by seeds of sobemoviruses has been reported, it does not constitute a conserved mechanism in the genus, and the results of the present study seems to support those of previous works.

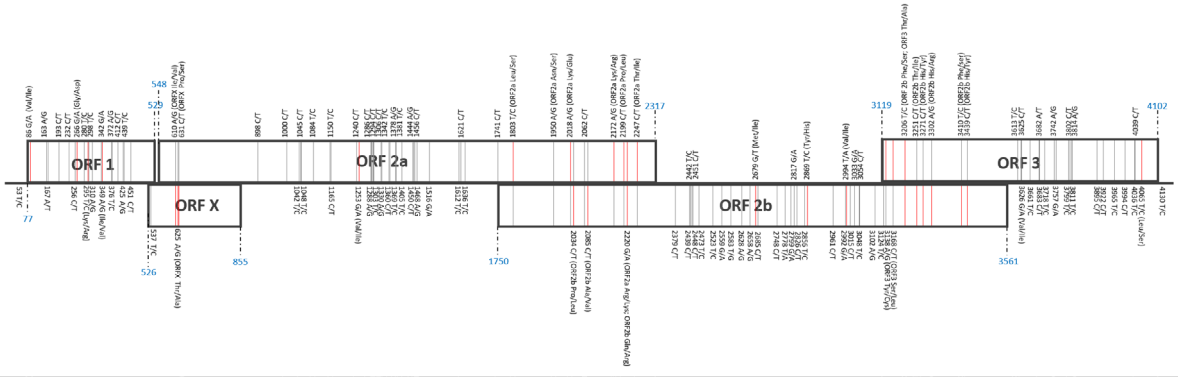

Figure 2 - Sequences comparisons between genomes of physalis rugose mosaic virus (PhyRMV) Brazilian isolates. Map of nucleotide and amino acid substitutions in PhyRMV genome. Nucleotide and amino acids substitution are indicated following the order: isolated characterized in this study/Piracicaba isolate. Red lines indicate nonsynonymous nucleotide substitutions (dN) and grey lines indicate synonymous nucleotide substitutions (dS). Nucleotide positions are numbered according to sequence of the PhyRMV characterized in this study (MN782300).

Table 1 - Transmission rate of seeds to *Physalis peruviana* seedlings.

Experiment	---------Infectivity (% infected plants)[a]----------		Transmission rate (% infected seedlings) [b]
	C-	C+	
1	0/10 (0)	20/20 (100)	0/200 (0)
2	0/10 (0)	20/20 (100)	0/200 (0)
3	0/10 (0)	20/20 (100)	0/200 (0)

[a] Number of infected plants/number of inoculated plants, verified by RT-PCR amplification of viral genomic fragments at 30 days after inoculation; *P. peruviana* inoculated only with buffer were used as a negative control. C- and C+ correspond to the negative and positive controls, respectively. From the plants used as a C+, seeds were collected for the transmission test;
[b] Number of infected seedlings/ number of seedlings evaluated, verified by RT-PCR amplification of viral genomic fragments at 50 days after the emergency.

Several factors may be responsible for the virus inability to be transmitted by seeds. Konaté et al. (2001) demonstrated that the sobemovirus RYMV is seed-borne but not transmitted by rice seeds and that this inability is due to the inactivation of the virus during seed maturation. The non-transmission of a virus may also be due to the activity of the vascular tissue and the location of the pathogen in the seed (ASSIS FILHO; SHERWOOD, 2000). Additionally, environmental factors such as temperature, the viral isolate, the host, and stage of infection can also influence the ability of the virus to be transmitted via seeds (SASTRY, 2013).

Results presented here increase our knowledge regarding the molecular characterization of an emerging viral species, provide the second near-complete genome sequence of PhyRMV, and showed evidences that PhyRMV isolated from southern Brazil is not transmitted by physalis seeds. Future studies should be performed to determine the possibility of PhyRMV transmission by an insect vector, as well as its virus-vector relationship.

ACKNOWLEDGEMENT

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil – Finance code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 437059/2018-9), and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina.

DECLARATION OF CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest. The funding entities had no influence on the study design; nor in the collection, analysis or interpretation of the data; in the writing of the manuscript, nor in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

FNS, ESG and AS conceived and designed experiments. EGS, AS, SCN, and CBF performed the experiments; TVMF performed double-strand RNA extraction; ANJ, FNS and AS performed sequence analysis and bioinformatics; FNS, RTC and AB supervised and coordinated the greenhouse experiments and data analysis; FNS and AS prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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