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RESEARCH ARTICLE

JOHNSONGRASS MOSAIC VIRUS INFECTING SORGHUM IN BRAZIL

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ABSTRACT

Sorghum *bicolor* (L.) Moench is cultivated in several tropical and subtropical regions in the world. Among the diseases, the mosaic caused by potyvirus is an important constraint for the agricultural production causing reduction in grain and forage sorghum production.In Brazil, only *Sugarcane mosaic virus* (SCMV) had previously been reported as the potyvirus species causing mosaic in sorghum and maize. A survey was carried out in sorghum plantations of the State of Minas Gerais, Brazil, during the 2014/2015 crop season for monitoring mosaic disease. Samples of sorghum plants expressing virus disease symptoms were collected for molecular analyzes. Molecular characterization of coat protein (CP) of the potyviruses naturally infecting sorghum, allowed us to identify the *Johnsongrass mosaic virus* (JGMV) as a new causal agent of mosaicdisease in sorghum in Brazil. The sequences of the Brazilian JGMV sorghum-infecting (JGMV-Sr) isolates were deposited in the GenBank under the accession numbers KY952241, KY952242, and KY952243. Comparisons of the CP gene sequences of these Brazilian JGMV-Srisolates revealed high nucleotide (nt) and amino acid (aa) sequence identities, ranging from 97.93 to 98.23%, and 99.12 to 99.20%, respectively, with the U07218.1 (JGMV-MDKS1) isolate. The Brazilian JGMV-Sr isolates were distinct from the Brazilian forage grasses-infecting (JGMV-Fg) isolates (KT833782 and KT289893).Transmission evaluations showed susceptibility of the teosinte, *Sorghum verticilliflorum* and *Sorghum bicolor* (L.) Moench, except line QL3.Maize and sugarcane genotypes were not infected by the Brazilian JGMV-Sr isolate. However, it is important to test more genotypes. This is the first report showing the identification and molecular characterization of the JGMV species naturally infecting sorghum at field conditions, expanding the knowledge about the dynamic and range of the mosaic causal agent for this crop in Brazil.

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INTRODUCTION

Sorghum *bicolor* (L.) Moench is cultivated in several tropical and subtropical regions in the world, and is the fifth major cereal in terms of production and acreage, after wheat, rice, maize and barley (FAO, 1999). Sorghum has water stress adaptive characteristics, which explains its cultivation in millions of hectares, in countries from Africa, Asia, Oceania and Americas (FAO, 1999). Among the diseases, the viruses are important constraint for the agricultural production worldwide, and the mosaic caused by potyviruses is widely disseminated affecting *Poaceae* species. In susceptible sorghum cultivars, mosaic causes reduction in grain and forage production, and the symptoms can be expressed as typical mosaic or leaf necrosis depending on the genotype (Silva *et al.*, 2012). Six species of potyviruses have been identified worldwide, causing mosaic symptoms in cultivated andweed grasses, including: *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), *Maize dwarf mosaic virus* (MDMV), *Johnsongrass mosaic virus* (JGMV) (Shukla *et al.*, 1994), *Zea mosaic virus* (ZeMV) (Sheifers *et al.*, 2000), and *Pennisetum mosaic virus* (PenMV) (Deng *et al.*, 2008). In Brazil, mosaic in sorghum has as causal agent the SCMV species which strain constitutes a monophyletic group distinct from the others identified worldwide so far (Souza *et al.*, 2012). Silva *et al*

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(2016) and Camelo-Garcia *et al.* (2016) identifiedisolates of the JGMV species infecting forage grasses, *Pennisetumpurpureum* and *Panicum maximum*in Brazil, deposited in GenBank under numbers KT833782 and KT289893, respectively. However, none of these two isolates were identified naturally infecting maize or sorghum at field conditions. In addition, there is evidence of the presence of potyvirus species in maize whose identification is still necessary (Oliveira *et al.*, 2005). Maize aphid, *Rhopalosiphum maidis* (Fitch, 1856) (Hemiptera: Aphididae) is the most efficient vector in potyviruses transmission, and in Brazil is found mainly in regions where sorghum and maize are grown on the second harvesting (off-season), causing economic damages (Goussain, 2001). However, the green aphid, *Schizaphis graminum* (Rondani, 1852) (Hemiptera: Aphididae), considered an important pest of sorghum, also transmits the viruses (Cruz and Vendramin, 1995). The succession of grasses, maize and sorghum, and the increased incidence of vector in the off-season, are factors that contribute for the dissemination of the mosaic disease in both crops. Losses of up to 50% in production can be caused by this virosis (Waquil *et al.*, 1996). A survey was carried out in sorghum plantationsof the State of Minas Gerais, Brazil, during the 2014/2015 crop seasonfor monitoring mosaic disease.Samples of sorghum plants expressing virus disease symptoms were collected for molecular analyzes.Among the samples we identified JGMVisolates infecting grain and forage sorghums [Sorghum bicolor (L.) Moench], respectively, from Paracatu and Felixlândiamunicipalities. Here, we are reporting for the first time the occurrence of the JGMV species naturally infecting sorghum crops under field conditions in Brazil.

mosaic symptoms (i.e., small chlorotic areas interspersed with green areas (Figure 1 A-C) had their foliar tissue collected and the viruses identified through molecular analyzes. The molecular identification of theviralspecies had two steps, first the RNA was extracted from the leaf tissue using the RNeasy®Plant Mini Kit (Qiagen) and cDNA synthesized from 1.0 µg total RNA using oligo$(dT)_{18}$ and the SuperScript®III First-Strand Synthesis System (Invitrogen). In the first step, primers that amplify the conserved region of the CP of potyviruses were used PZEO1 x PZEO2 (Seifers *et al.*, 2000). Then, the positive samples were submitted to PCR reactions with primers specific for the region that encompasses the CP and a partial sequence of the nuclear inclusion protein (NIb) for all the six potyviruses from the mosaic complex (Table 1 fromSouza and Barros 2016). The PCR reactions were also performed according to Souza and Barros, 2016. The primers set combinations PJG_F X PSR (Jiang and Zhou, 2002), and PJG_F (Jiang and Zhou, 2002) X PJG_R (design by the author Barros, B.A.) (Table 1) resulted in amplification of the JGMV in the samples with fragment sizes of the 813 bp and 414 bp, respectively. These primer sets amplify the N-terminal portion of the CP, allowing the identification of the JGMVisolatesby sequencing (Shukla *et al.*, 1989). Amplicons were purified using ExoSAP-IT for PCR Product Cleanup (USB) and sequenced. The obtained sequences were analyzed using the Sequencher 1.4.1 software, performed by a two-step PCR. Sequence similarity searches against the NCBI sequence database (GenBank), allowed the identification of the JGMV species as the causal agent of the sorghum mosaic disease in Brazil. The JGMV isolate 7 infecting grain sorghum and the isolates 48 and 49 infecting forage sorghum were collected in

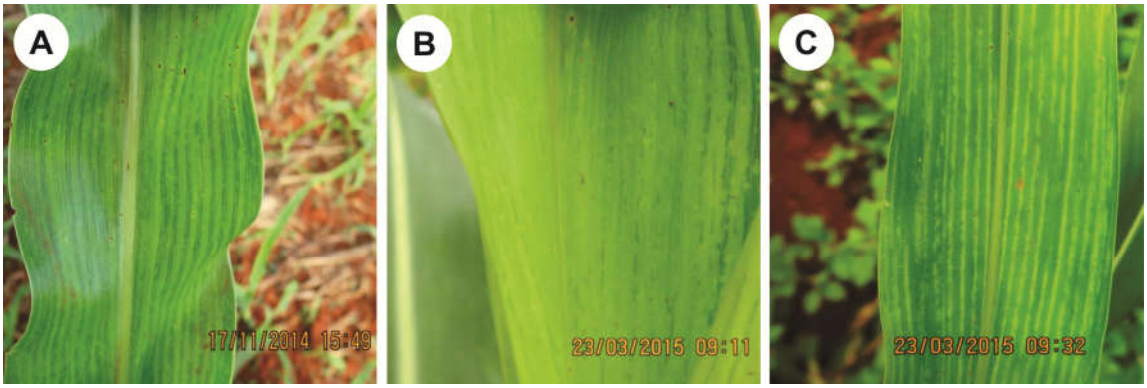

Figure 1. Mosaic symptoms in the leaves of grain sorghum (A), and forage sorghums (B and C) under field conditions, respectively, collected during the survey in Paracatu and Felixlândia municipalities of the Minas Gerais State (MG), Brazil

Table 1. Sequences of primers used in RT-PCR analysis

Potyvirus	Primer Identification	Sequence5' – 3'[a]	Reference
SCMV, SrMV, MDMV and JGMV	PSR[c]	CAGCTGTGTGBCKSTCTGTATT	JIANG &ZHOU (2002)
JGMV	PJG[b]-F	AAACCAGCTAGTGGTGAAGGC	JIANG & ZHOU (2002)
JGMV	PJG[d]-R	CACCAGACCATTAATCCGTTC	Design by author Barros, B. A.

[a]– B= (G, C ou T); K = (G ou T); S = (G ou C); N = (A, G, C ou T); Y = (C ou T); R = (A ou G).
[b]– Fowardprimer specific for determined potyviruses.
[c]– Reverse primer common for all potyviruses.
[d]–Reverse primer design by author Barros, B.A.

MATERIALS AND METHODS

Leaf sampling and virus identification

During the survey for common mosaic viral disease in sorghum crops carried out in the state of Minas Gerais (MG, Brazil) in the 2014/2015 crop season, plants expressing typical

the municipalities, respectively, Paracatu and Felixlândia, both in the state of Minas Gerais, Brazil.Sequences of the gene and the translated coat protein (CP) of theseisolates were deposited in GenBank under the accession numbers KY952241(isolate 7), KY952242 (isolate 48) and KY952243 (isolate 49).

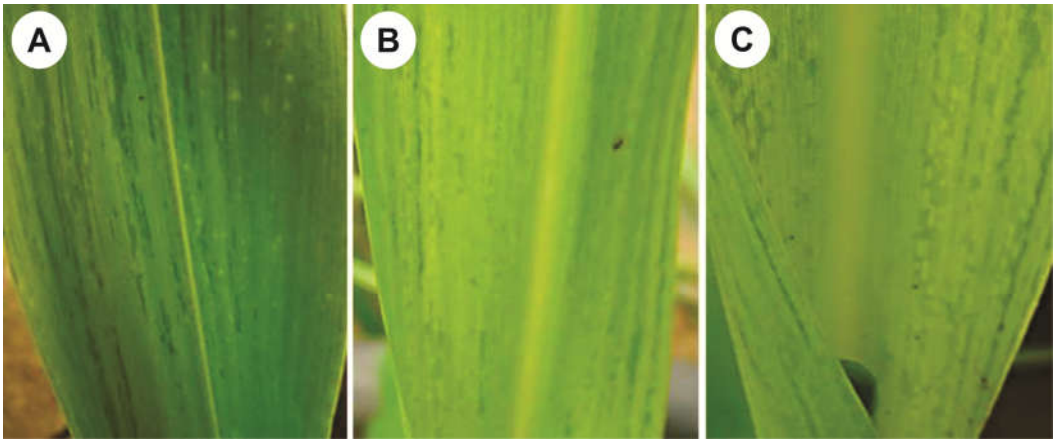

Figure 2. Mosaicsymptoms induced in leaves of the plant species mechanically inoculated with Brazilian JGMV-Sr isolate: Sorghum BRS 506 (A), *Euchlaena mexicana* (B), and *Sorghum verticilliflorum* (C)

Table 2. Pairwise percent coat protein nucleotide sequence identity (above diagonal) and amino acid (below diagonal) sequence identity among Brazilian JGMV-Sr isolates, Brazilian JGMV-Fg isolates and USA isolate of JGMV. The GenBank accessions numbers are followed by the host species and country name or abbreviation

GenBankAccessions	U07218.1	KY952241	KY952242	KY952243	KT289893	KT833782
U07218.1 MDKS1-USA	-	98.23	97.93	98.01	79.18	78.82
KY952241-Sorghum_7-Brazil	99.20	-	98.82	98.74	77.36	76.43
KY952242-Sorghum_48-Brazil	99.12	100.00	-	99.71	79.09	78.50
KY952243-Sorghum_49-Brazil	99.16	100.00	100.00	-	78.12	77.14
KT289893-Panicum maximum -Brazil	81.52	81.20	84.51	83.12	-	95.87
KT833782-Pennisetum purpureum-Brazil	80.94	80.80	84.96	82.70	96.53	-

Mechanical transmission of the Brazilian JGMV-Sr isolate

The study was conductedat Embrapa Maize and Sorghum, experimental station, Sete Lagoas, Brazil, andcarried out in a greenhouse with preventive insecticide applications to avoid the occurrence of insects. The experiment was conductedin pots filled with 25 kg of a Latosol, typical Brazilian Cerrado soil.Soil pH was corrected by liming and the fertilization was performed according to soil analysis. A rate equivalent to 400 $kgha^{-1}$ of the formula 08-28-16 + 0.3% Zn and 40 $kgha^{-1}$ of FTE BR12 was mixed to the soil in the pots. The experimental unit constituted of one pot containing five seedlings/pot. In thetransmission test were evaluated *Sorghum bicolor* (L.) Moench: varietiesBRS Ponta Negra and BRS 506, and the lines BRS 007B, ATF54, QL3, 9910032, 9929020, *Zea mays* L.:inbred lines L18, L19, L520, L3, and L2841 and a commercial hybrid; *Saccharum*spp: RB928064 and RB855536,besides *Sorghum verticilliflorum*and *Euchlaena mexicana*. Treatmentsconsisted of the (i) inoculum of the Brazilian JGMV-Sr isolate, and (ii) a negative control for inoculation (mock treatment). The presence of the JGMV was confirmed molecularly and the inoculumwas prepared using leaves of the symptomatic plants macerated in cooled phosphate buffer (10 mM, pH 7.0), at a ratio of 1:3 (weight/volume) (Souza *et al.*, 2008). Carborundum 600 mesh (Sigma-Aldrich) was added to the inoculum solution, which was kept on ice throughout the inoculation process. The first inoculation was performed in the middle to the basal part of the leaveswhen the seedlings had three to four leaves. The same mechanical procedure was adopted for the mock treatment using phosphate buffer (10 mM, pH 7.0) containing 600 mesh carborundum. Two inoculations were performed with a weekinterval. Phenotypic evaluations were initiated one week after the first inoculation and were repeated weekly along one month and five days period. Phenotypes were evaluated based on mosaic symptoms and classified for the presence and absence (Figure 2).

Pairwise sequence alignment and phylogenetic analysis

In our analysis, we used a data set of32 JGMV CP nucleotide sequences including isolates downloaded from NCBI database, and our three Brazilian JGMV-Srisolates. Additionally, five other species as outgroups: *Sugarcane mosaic virus* (SCMV_KR108212.1), *Maize dwarf mosaic virus* A (MDMV-A_U07216.1), *Zea mosaic virus* (ZeMV_AF228693.1), *Pennisetum mosaic virus* (PenMV_JX070151.1), and *Sorghum mosaic virus* (SrMV_KM025045.1). Apart from JGMV isolates described here, the sequences were downloaded from the NCBI database and comprised the complete ORF of the protein or were at least 700 base pairs (bp) in length. Alignments were obtained and conferred using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MUSCLE (executed in MEGA7 - Kumar, Stecher and Tamura, 2016). Pairwise comparisons were performed using SDT v. 1.2 (Muhire *et al.*, 2014). Phylogenetic reconstruction was carried out with MEGA7 (Kumar, Stecher, andTamura, 2016) based on the Neighbor-Joining method (Saitou and Nei, 1987).The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The tree was generated using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and the percentage of replicate trees in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985).

RESULTS

Molecular characterization, sequencingand phylogenetic analysis of Brazilian JGMV-Sr isolates

Brazilian JGMV-Sr isolates showed the highest percentage of identities at coat protein nucleotide and amino acid sequences

with the JGMV-MDKS1, which values ranged from 97.93 to 98.23%, and 99.12 to 99.20%, respectively (Table 2). When compared with the Brazilian JGMV-Fg isolates (KT833782 and KT289893), these values ranged from 76.46 to 79.09%, and 80.80 to 84, 96%.

Alignmentsof the deduced amino acid (aa) sequences of the CP N-terminal showed the Brazilian JGMV-Sr isolates (KY952241, KY952242, and KY952243) and the JGMV-MDKS1strain (U07218.1) with five aa residues lessthan Brazilian JGMV-Fgisolates (KT833782 and KT289893), residues 21, 32, 33, 45 and 46 (Figure 3). Also these two latter isolates showed several amino acids substitutions, as the MVWCIENG potyvirus conserved motif of the CP core was changed to MVWAIENG in relation to theBrazilian JGMV-Sr isolates, and JGMV-MDKS1 strain from USA.

the other composed only by Brazilian JGMV-Fgisolates (KT833782, KT289893 and KC333416.1). Additionally, a third clade, grouped JGMV isolates from Australia.

Mechanical transmission of the Brazilian JGMV-Sr isolate

Among thesorghum genotypes, except the line QL3, the varieties BRS Ponta Negra and BRS 506, and lines BRS 007B, ATF54, 9910032, and 9929020 were infected and expressed the typical mosaic symptoms. Also, the *Poaceae* species *Euchlaena mexicana* and weed *Sorghum verticilliflorum* were infected and showed similar symptoms. All the fivemaize genotypes, inbredlines L18, L19, L520, L3, and L2841, and the two *Saccharum* spp.genotypes, varieties RB928064 and RB855536, were not infected. Variations in relation to mosaic symptoms expression were verified: (i) mosaicmore confined to longitudinal sectors as observed at field (Figure 1A and C),

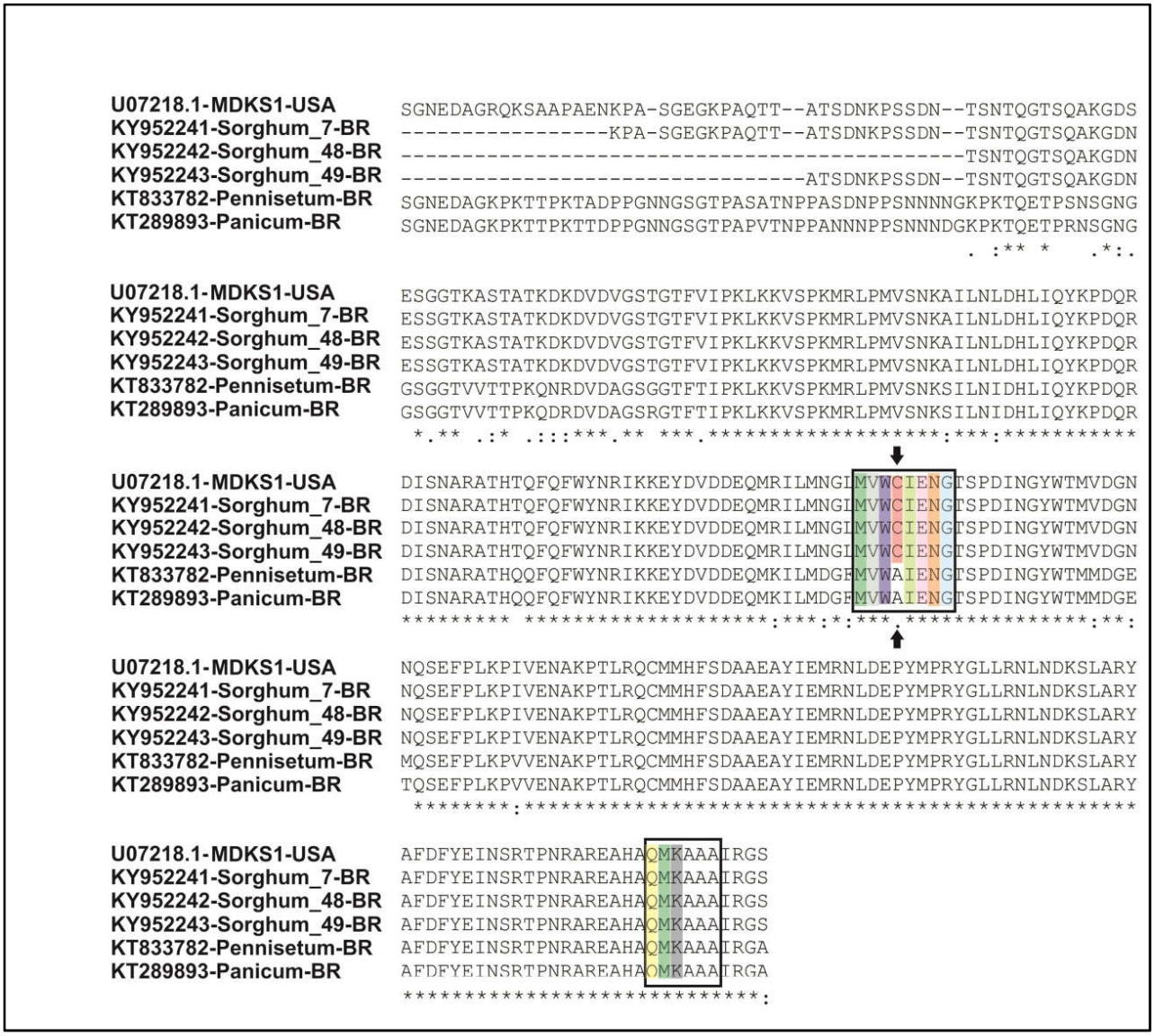

Figure 3. Multiple alignments of CP N-terminal amino acid sequences of JGMV strains from NCBI database and Brazilian JGMV-Srisolates from this work. The accession numbers are followed by host plant species and country abbreviation, BR=Brazil and USA= United States of America. The conserved motifs (MVWCIENG, QMKAAA) of the potyviruses CP core are highlighted inside a box and the position with divergent amino acids is demarcaded by black arrows

The phylogenetic tree (Figure 4) and color-coded pairwise identity matrix (Figure 5) showed the JGMV accessions from Brazil divided into two groups, one composed by the JGMV-Sr isolates (KY952241, KY952242 and KY952243) plus theJGMV isolates from USA (U07218.1 and U07217.1), and

and greenhouse conditions, BRS 506 mechanically inoculated (Figure 1D), and (ii)Typicalmosaic, chlorotic areas interspersed with green onesexhibited at field (Figure 1B) and greenhouseconditions, Euchlaena *mexicana* and weed *Sorghum verticilliflorum*mechanically inoculated (Figure 2B and C).

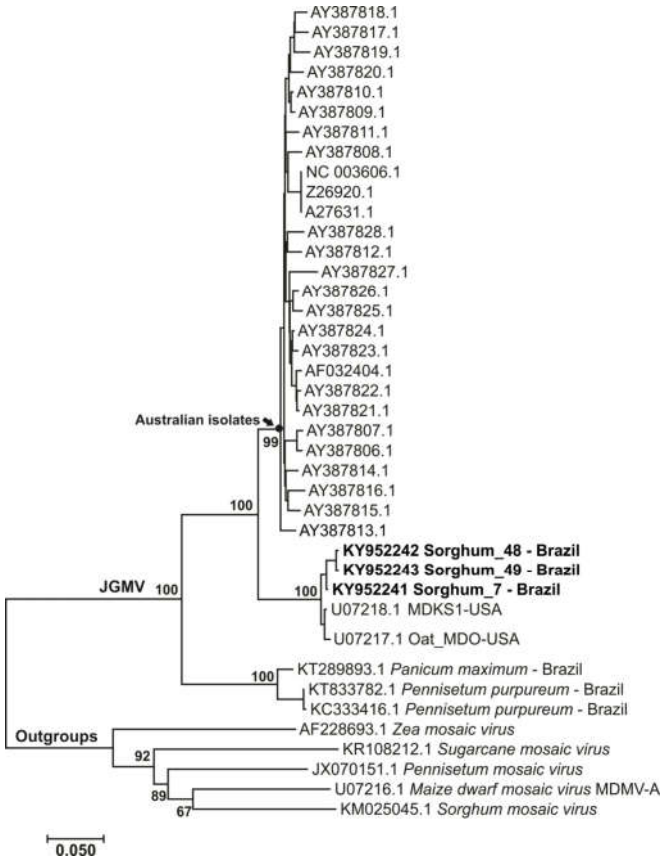

Figure 4. Phylogenetic tree derived from the CP nucleotide sequences of the 32 *Johnsongrass mosaic virus* (JGMV), plus five outgroups potyviruses. The tree was generated using MEGA 7 by the neighbor-joining method with 1,000 bootstrap replicates. Bootstrap values are shown at the internodes, and NCBI accession numbers are indicated.The Brazilian JGMV-Sr isolates from this study are in bold

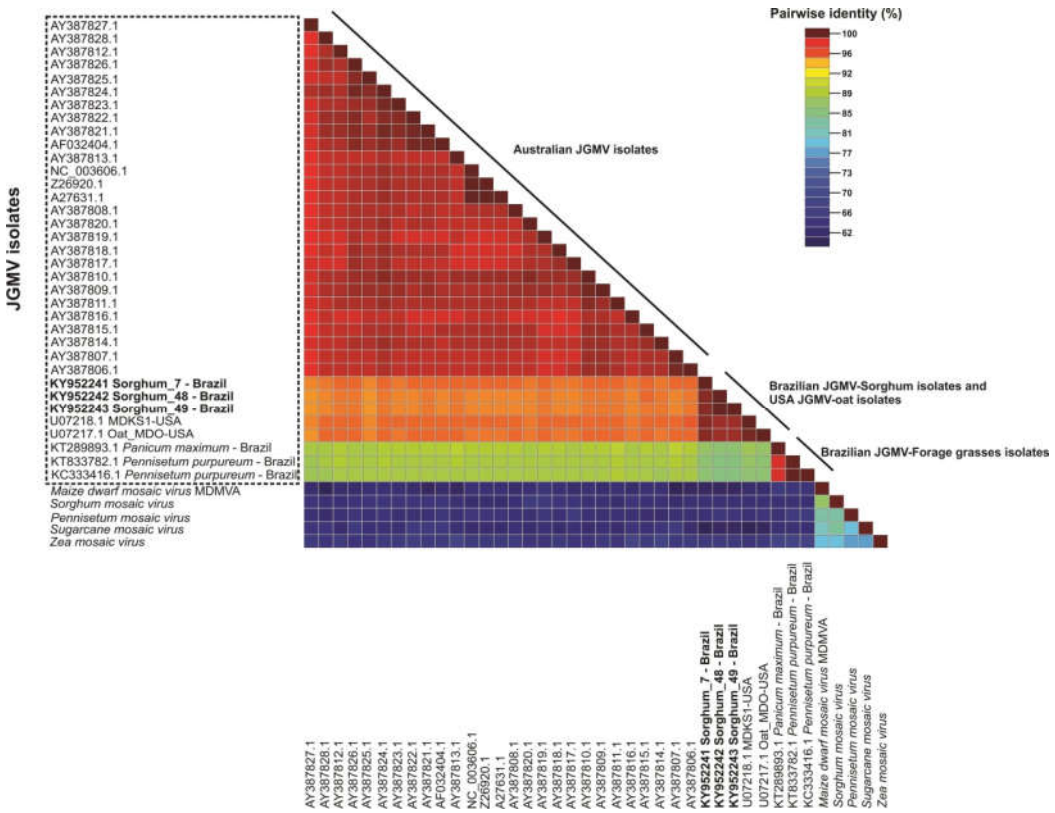

Figure 5. Color-coded pairwise identity matrix based on the CP nucleotide coding sequences of Brazilian JGM-Sr isolates from this study, and 32 JGMV plus five outgroups potyviruses downloaded from GenBank. Each colored cell represents a percentage identity score between two sequences. A color key indicates the correspondence between pairwise identities and the colors displayed in the matrix

DISCUSSION

Distinct variations among the Brazilian JGMV-Sr isolates (GenBank KY952241, KY952242, and KY952243), and the Brazilian JGMV-Fg isolates, *Panicum maximum* (GenBank KT289893) and *Pennisetum purpureum* (GenBanK KT833782), were demonstrated through DNA sequencing and alignment of the CP sequences, with average percentage identities of 77,77% and 82.88% at the nucleotide and correspondent amino acid sequences, respectively (Table 2 and Figure 4). The nucleotide identities of the Brazilian JGMV-Sr isolates meet the criteria for JGMV *Potyvirus* species classification due identity above the 76-77% for the CP (Adams *et al.*, 2012). As mention by Shukla *et al*. (1991) the correct identification of a pathogen is the first step toward studies to allow its eventual control. The primers pair described by Camelo-Garcia *et al*. (2016) and Silva *et al*. (2016) to amplify the CP gene sequences of the Brazilian JGMV-Fg isolates from *Panicum maximumand Pennisetum purpureum*, respectively, does not produce amplicons using the BrazilianJGMV-Sr isolates. It isdue variations caused by a mismatch at the 3` end of their forward primer (CAAGCCC CATACTTGTCGG), whereonly the underlined nucleotide sequence hascomplementarysequence to Brazilian JGMV-Srisolates.In addition, the primers pair used by Lenardon and Giolliti (2004) to identify the JGMV in Argentinawas not able to amplify the same fragment from samples infectedwith the Brazilian JGMV-Sr isolates. The highly conserved motifin the CPs potyviral, MVWCIENG (Pappu *et al*., 1993) was preserved in the Brazilian JGMV-Sr isolates, and "C" was replaced by "A", MVWAIENG,in the Brazilian JGMV-Fg isolates (Figure 3). The N-hypervariable region of the Brazilian JGMV-Sr isolatesand JGMV-MDKS1hasa dinucleotide repeat $(CA)_6$, as part of the deduced ATHTQ peptide sequence of the CP, and isfive amino acids shorter than Brazilian JGMV-Fg isolates. Zhao *et al.* (2011) found in potyvirusesgenome the predominance of the repeats AC/CA, AG/GA and AAG/GAA, and mentioned that microsatellites may have many important functions in terms ofgenomic organization and species evolution. A high mutation frequency of microsatellites allow to increase the diversity inpathogen populations (Pearson *et al.*, 2005), and some of them may promote potyviruses species evolving to adapt to their hosts (Zhao, 2011).Majority of the CP aminoacid substitutions was verified in the amino terminal regions among the Brazilian JGMV-Sr and JGMV-Fg isolates. It was also observed by Shukla *et al.* (1991) when comparing U07218.1 (JGMV-MDKS1) and U07217.1 (JGMV-MDO) from USA with the JGMV-JG, a Johnsongrass-infecting isolate from Australia. Several differences in the CP nucleotide sequences among the Brazilian JGMV-Srand JGMV-Fg isolates (Figure 3) grouped them indistinct monophyleticsubgroups (Figure 4). The third and the largest cluster composed by JGMV isolates from Australia showed higher pairwise identities than the other two groups (Figure 4 and 5).

Through transmission test applying the Brazilian JGMV-Sr isolate, we were able to verify mosaic symptoms and infection in teosinte (*Euchlaena mexicana*), *Sorghum verticilliflorum*, and sorghum genotypes, except QL3, with the virus been detected by RT-PCR. All maize and the two sugarcane genotypes were not infected in the transmission evaluations. Persley *et al* (1981) found that most of the maize hybrids in Australia had at least good field JGMV resistance, besides a high correlation between ratings of resistance verified from greenhouse and natural field infection. In addition, Camelo-Garcia *et al.* (2016) showed that the Brazilian JGMV-Fg isolatefrom *Panicum maximum* cv Mombaça, did not infected maize. Silva *et al.* (2016) showed through transmission evaluations that the Brazilian JGMV-CNPGL (KT833782) isolated from Elephant grass (*Pennisetum purpureum*) was capable to infect maize, suggesting a potential threat to thiscrop in Brazil. However, so far, this strain was not identified naturally infecting maize at field. Also, the KITC motif of HCPro involved in the interaction of the viral particle and the stylus (Blanc *et al.*, 1998), was absent from the JGMV-CNPGL isolate (Silva *et al.*, 2016). On the other hand the vegetative propagation of the Elefant grass by stem cuttings (Pereira *et al.*, 2017), allows the viraltransmission without the presence of the vector. Seifers *et al.* (2005) using transmission test demonstrated that JGMV-MDO (U07217.1), which also shares high identity with the Brazilian JGMV-Sr isolates, was capable to infect *Sorghum bicolor*, except line QL3 genotype, as found in this work. Originally, thisJGMV-MDO strain from USAwas described as MDMV oat-infecting strain (McDaniel and Gordon, 1989; McKern, 1990). Perennial wild *Sorghum verticilliflorum,* an important weed in many crop plantations in Brazil (Tessele *et al.*, 2014), was susceptible to the Brazilian JGMV-Sr isolates identified in this research.It is animportant factor in the intercropping periods allowing thesurvival of the JGMV between seasons acting as viral reservoirs. The most efficient way to control disease spreading is through the development of resistant cultivar. Studying JGMV resistance, Presley *et al*. (1981) observed ratios of maize segregating generations that were consistent with resistance being controlled by a single dominant gene, which is important to breeders toward introgression of disease resistance genes into susceptible cultivars.

Conclusion

The identification of *Johnsongrass mosaic virus* (JGMV) isolates naturally infecting sorghum (*Sorghum bicolor* (L.) Moench) at field level in Brazil is reported. These Brazilian JGMV-Sr isolates have high CP sequence identities with the USA JGMV strains, MDO_U07217.1 and MDKS1_U07218.1, and grouped with them in the phylogenetic tree. The transmission evaluations showed that*Sorghum bicolor*, except line QL3, teosinte and *Sorghum verticilliflorum* were infected and expressed mosaic symptoms. The sorghum line QL3 identified as a source of JGMV resistance could be used in breeding programs to develop new resistant cultivars.The Brazilian JGMV-Fgisolates previously identified infecting *Panicum maximum* (GenBank KT289893)and *Pennisetum purpureum* (GenBanK KT833782),aredifferent from that identified in this study, and so far, were not identified naturally infecting sorghum crops. Maize and sugarcane genotypes used in the transmission test were not infected by the Brazilian JGMV-Sr isolates described in this study.However more genotypes, and also more weed poaceas will be evaluatedlooking for host range of the Brazilian JGMV-Sr isolates.

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