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Detection of *Badnavirus* in pineapple in northeastern Brazil

Detecção de *Badnavirus* em abacaxizeiro no Nordeste do Brasil

Edlene Maria da Silva Moraes Santos[1]; Jaqueline Figueredo de Oliveira Costa[2]; Mayra Machado de Medeiros Ferro[3]; Sarah Jacqueline Cavalcanti da Silva[4]; Iraildes Pereira Assunção[5]; Gaus Silvestre de Andrade Lima[6]

[1]ORCID: https://orcid.org/0000-0003-4222-0805, Universidade Federal de Alagoas (UFAL), Pesquisadora do Campus de Engenharias e Ciências Agrárias, Rio Largo - AL, BRAZIL. E-mail: edlenemsm@hotmail.com
[2]ORCID: https://orcid.org/0000-0003-0121-699X, Universidade Federal de Alagoas (UFAL), Pesquisadora do Campus de Engenharias e Ciências Agrárias, Rio Largo - AL, BRAZIL. E-mail: jaquelinefigueredo@hotmail.com
[3]ORCID: https://orcid.org/0000-0002-4362-8127, Universidade Federal de Alagoas (UFAL), Pesquisadora do Campus de Engenharias e Ciências Agrárias, Rio Largo – AL, BRAZIL, E-mail: mayra.ferro@hotmail.com
[4]ORCID: https://orcid.org/0000-0001-9336-4126, Universidade Federal de Alagoas (UFAL), Profª Drª do Campus de Engenharias e Ciências Agrárias, Rio Largo – AL, BRAZIL. E-mail: sarah.silva@ceca.ufal.br
[5]ORCID: https://orcid.org/0000-0001-5087-0168, Universidade Federal de Alagoas (UFAL), Profª Drª do Campus de Engenharias e Ciências Agrárias, Rio Largo - AL, BRAZIL. E-mail: iraildes.assuncao@ceca.ufal.br
[6]ORCID: https://orcid.org/0000-0003-2910-5896, Universidade Federal de Alagoas (UFAL), Prof Dr do Campus de Engenharias e Ciências Agrárias, Rio Largo – AL, BRAZIL. E-mail: gaus@ceca.ufal.br

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SUMMARY: O Brasil é um dos principais produtores mundiais de abacaxi (*Ananas comosus* L. Merril), sendo a região Nordeste destaque em âmbito nacional. O abacaxizeiro está exposto a diversos problemas fitossanitários, inclusive as viroses. As badnaviroses do abacaxizeiro são causadas por duas espécies distintas: *Pineapple bacilliform CO virus* (PBCoV) e *Pineapple bacilliform ER virus* (PBErV). O presente estudo teve como objetivo detectar possíveis espécies do gênero *Badnavirus* em amostras de abacaxizeiro no Nordeste do Brasil, via PCR e sequenciamento da região RT/RNaseH. Amostras foliares de abacaxizeiros foram coletadas nos estados de Alagoas, Maranhão, Paraíba e Pernambuco e, posteriormente, submetidas à extração de DNA total e amplificação via PCR. Amostras positivas para *Badnavirus* foram selecionadas para sequenciamento. Análises de comparações de sequências pareadas revelaram que todas as sequências obtidas neste trabalho apresentaram identidade superior a 80% com a sequência da espécie PBCoV (EU377664), proveniente da Austrália, corroborando integralmente com as análises filogenéticas. Estes resultados sugerem a ampla disseminação do PBCoV no Nordeste brasileiro e registra o primeiro relato de *Badnavirus* na cultura do abacaxizeiro no Brasil.
KEYWORDS: *Caulimoviridae*, *Ananas comosus*, análise molecular.

ABSTRACT: Brazil is one of the main global producers of pineapple (*Ananas comosus* L. Merril), with emphasis in the Northeastern region of the country. Pineapple is exposed to several phytosanitary problems, including viruses. Pineapple badnaviruses are caused by two distinct species: *Pineapple bacilliform CO virus* (PBCoV) and *Pineapple bacilliform ER virus* (PBErV). The present study aimed to detect possible species of the genus *Badnavirus* in pineapple samples in Northeastern Brazil, via PCR and sequencing of the RT/RNaseH region. Leaf samples of pineapples were collected in the states of Alagoas, Maranhão, Paraiba and Pernambuco, and subsequently subjected to total DNA extraction and amplification via PCR. *Badnavirus* positive samples were selected for sequencing. Analysis of pairwise comparisons revealed that all sequences obtained in this work showed an identity greater than 80% with the sequence of the species PBCoV (EU377664), from Australia, fully corroborating with phylogenetic analyzes. These results suggest the widespread of PBCoV in Northeastern Brazil and record the first report of *Badnavirus* in pineapple culture in Brazil.
KEYWORDS: *Caulimoviridae*, *Ananas comosus*, molecular analysis.

INTRODUCTION

Pineapple (*Ananas comosus* L. Merril) is a perennial, herbaceous monocotyledon, belonging to the family Bromeliaceae, with approximately 50 genera and 200 species. From the economic point of view the genus *Ananas* is the most important in the family (CUNHA & CABRAL, 1999).

Brazil is one of the biggest pineapple producers in the world, with a production ranging from 2.2 to 2.7 million tons in the last decade (MATOS, 2018). In 2018, the Northeastern Brazilian region produced 590 thousand tons of pineapple, with the state of Paraiba standing as the biggest national producer (IBGE, 2018). Despite the high volume of pineapple produced in Brazil and in the world, this crop is exposed by many phytosanitary problems causing significant economic losses (MATOS et al., 2009), and viral diseases are included amongst these.

The most important pineapple virus are *Pineapple mealybug wilt associated viruses* (PMWaVs; genus *Ampelovirus*, family *Closteroviridae*) and viruses of the genus *Badnavirus* (family *Caulimoviridae*) (GAMBLEY et al., 2008a, b; SETHER & HU, 2002).

The family *Caulimoviridae* includes plant viruses with semicircular double-strand DNA (dsDNA) genome, 7.2-9.2 kbp in length, encapsidated into isometric or bacilliform particles, which replicated via an intermediary RNA (pararetrovirus) (GEERING & HULL, 2012; TEMIN, 1985). Pararetrovirus do not encode an integrase protein, however, they show capacity of integration inside the host genome for replication, which are known as endogenous pararetroviral sequences (Endogenous Pararetroviral Sequences - EPRVs) (GAYRAL & ISKRA-CARAUNA, 2009). Based in the host range, the type of vector, genome organization and phylogenetic relationships, this family is divided into the genera *Badnavirus*, *Caulimovirus*, *Cavemovirus*, *Dioscovirus*, *Petuvirus*, *Rosadnavirus*, *Solendovirus*, *Soymovirus*, *Tungrovirus* and *Vaccinivirus* (SUKAL et al., 2018; BATH et al., 2016; DIAZ-LARA & MARTIN, 2016).

The genus *Badnavirus* in the most diverse within the family, with 59 recognized species by the International Committee on Taxonomy of Viruses *(ICTV*, https://talk.ictvonline.org/taxonomy/*). The transmission occurs mainly by mealybugs (some by aphids)* in a semi-persistent manner (BATH et al. 2016; GEERING & HULL, 2012) and is among the most important plant viruses groups with a DNA genome. Badnaviruses are reported infecting a wide range of economically important tropical

crops such as rice (OMURA et al., 1983), sugarcane (LOCKHART & AUTREY, 1988), banana (LOCKHART & OLSZEWKI, 1993), cacao (KOUAKOU et al., 2012), citrus (AHLAWAT et al., 1996), pepper species (LOCKHART et al., 1997), yam (PHILLIPS et al., 1999), taro (YANG et al., 2003) and pineapple (GAMBLEY et al., 2008b).

In 1995 the first occurrence of *Pineapple bacilliform virus* was reported in pineapple hybrids in Australia (WAKMAN et al., 1995), then in Hawaii (SETHER & HU, 2002), China (WU et al., 2010) and Cuba (HERNANDEZ-RODRIGUEZ et al., 2013). At least two species of badnavirus infecting pineapple crops are known: *Pineapple bacilliform CO virus* (PBCoV) and *Pineapple bacilliform ER virus* (PBErV). Both species transmitted by the mealybug *Dysmicoccus brevipes* (Hemiptera: Pseudococcidae) and, in the case of PBCoV, also by the mealybugs *Planococcus citri* and *D. neobrevipes* (GAMBLEY et al., 2008; SETHER et al., 2012). An association between the symptoms and the infection by *Badnavirus* in pineapple was not yet demonstrated (GAMBLEY et al., 2008).

In this context, the objective of the present study was to detect species from the genus *Badnavirus* in pineapple samples from the Northeastern Brazil via PCR and sequencing of the RT/RNaseH region.

MATERIAL AND METHODS

Sampling, amplification of the RT/RNase/H region and sequencing

Pineapple leaf samples from five crop areas, showing typical symptoms of badnavirus infection (chlorotic streak) or asymptomatic, were collected in the states of Alagoas, Paraíba, Pernambuco and Maranhão (Table 1).

Table 1. Origin, cultivar, number and code of pineapple samples collected in the Brazilian Northeastern region. Sampling year: 2013.

Collection areas	Latitude	Longitude	Cultivar	No. of samples collected	Sample code
Arapiraca (AL)	09°45'09"	36°39'40"	'Jupi'	60	A1A a A60A
Pedras de Fogo (PB)	07°24'07"	35°06'59"	'Pérola'	60	A1PF a A60PF
São Domingos do Maranhão (MA)	05°34'33"	44°23'07"	'Pérola'	56	A1SD a A56SD
Coruripe* (AL)	10°07'32"	36°10'32"	'Pérola'	51	A1Pi a A51Pi
Pombos (PE)	08°08'29"	35°23'45"	'Pérola'	60	A1Po a A60Po

* The samples from the municipality of Coruripe were collected at Pindorama Village.

Total DNA extracted from each vegetal sample (DOYLE & DOYLE, 1990) was used as a template for amplification reactions via PCR. The oligonucleotide pair used in this work, BadnaFP (5'-ATGCCITTYGGIITIAARAAYGCICC-3') and BadnaRP (5'-CCAYTTRCAIACISCICCCCAICC-3') are based in the domain RT/RNaseH from the ORF 3 in the genome of various badnaviruses already described (YANG et al., 2003). PCR reactions were performed at a final volume of 30 µL, containing 3.0 µL buffer 10X PCR, 0.6 µL of a mixture of dNTPs 10 mM, 0.9 µL $MgCl_2$ 50 mM, 1.5 µL of each oligonucleotide at 10 µM, 10 ng of DNA template, and one unit of *Taq* DNA Polymerase, completing the volume with ultrapure H2O.

Amplification was performed with an initial denaturing step at 94°C for 4 minutes and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension step at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes. The presence of PCR products was confirmed by 1,2% agarose gel electrophoresis, purified using a kit GFX ™ PCR DNA and Gel Band Purification Kit (GE Healthcare), and sent for sequencing at Macrogen Inc. (Seoul, South Korea).

Analysis and comparison of sequences

The sequences obtained were assembled using the CodonCode *Aligner* v. 4.1.1 (www.codoncode.com) and, initially, analyzed using the algorithm BLAST*n* (ALTSCHUL et al., 1990) and GenBank non-redundant nucleotide database

(www.ncbi.nlm.nih.gov/genbank) to determine the viral species from which the sequences share higher identity. Pairwise comparisons between the obtained sequences and available sequences of badnaviruses in the GenBank (Table 2) were performed using *Sequence Demarcation Tool* v. 1.2 (MUHIRE et al., 2013) and the percent nucleotide sequences identity between isolates was determined.

Table 2. List of *Badnavirus* and *Tungrovirus* species used for pairwise sequence identity comparisons and phylogenetic analysis, with their respective acronyms and GenBank accession number.

Species	Acronym	GenBank acession number
Banana streak GF virus	BSGFV	AY493509
Banana streak OL virus	BSOLV	AJ002234
Banana streak MY virus	BSMYV	AY805074
Banana streak VN virus	BSVNV	AY750155
Bougainvillea chlorotic vein banding virus	BCVBV	EU034539 (=NC011592)
Cacao swollen shoot virus	CSSV	L14546 (= NC001574)
Citrus yellow mosaic virus	CiYMV	AF347695 (=NC003382)
Commelina yellow mottle virus	ComYMV	X52938 (=NC001343)
Dioscorea bacilliform SN virus	DBSNV	DQ822073 (=NC009010)
Pineapple bacilliform CO virus	PBCoV	EU377664
Pineapple bacilliform ER virus	PBErV	EU377672
Sugarcane bacilliform IM virus	SCBIMV	AJ277091 (=NC003031)
Sugarcane bacilliform MO virus	SCBMOV	M89923 (=NC008017)
Taro bacilliform virus	TaBV	AF357836 (=NC_004450)
*Rice tungro bacilliform virus**	RTBV	NC001914

* *Outgroup* sequence

Phylogenetic analysis

Sequences were aligned using the algorithm MUSCLE (EDGAR, 2004), manually set in the package MEGA6 (TAMURA et al., 2013) and submitted to Maximum Likelihood (MV) phylogenetic analysis (RIDLEY, 2006) using the nucleotide substitution model *General Time Reversible* with gamma distribution (GTR+G). The reliability of the generated tree was obtained from 2000 *bootstrap* repetitions. *Rice tungro bacilliform virus* (RTBV, genus *Tungrovirus*) was used as the *outgroup* (Table 2).

RESULTS AND DISCUSSION

A total of 287 samples were analyzed, with 24 of them being positive for badnavirus and amplification of fragments with the expected size (around 580 bb). Form these positive samples, 20 were selected for sequencing, from each sample collecting area, with a total of 100 samples, however, quality sequences were obtained only for 83 or them. Preliminary analysis using the algorithm BLAST*n* and comparing paired sequences with SDT revealed that sequences obtained in the present work have nucleotide identity above 80% with the isolate from the species *Pineapple bacilliform CO virus* – PBCoV (EU377664), from Australia (GAMBLEY et al., 2008b). This result shows that all sequences characterized in the present study, correspond to new isolates of PBCoV (Figure 1).

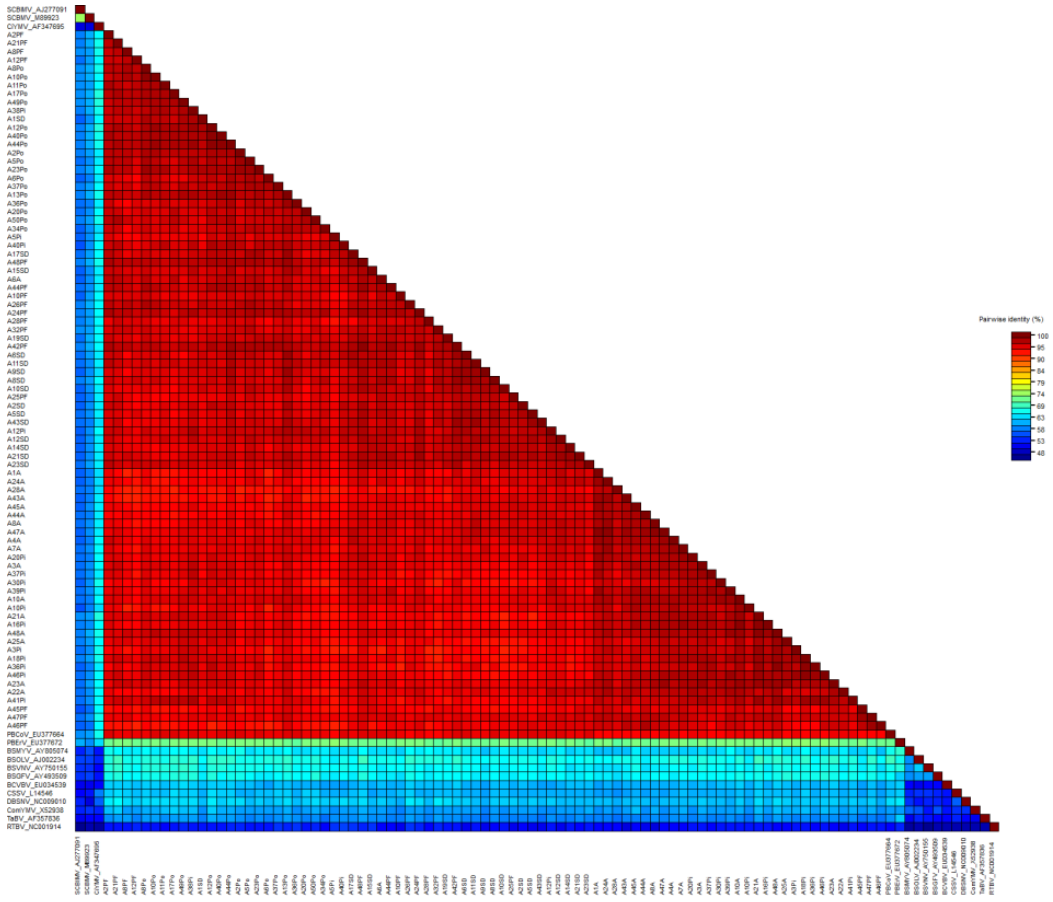

Figure 1. Pairwise comparison of the nucleotide sequences from the RT/RNaseH region from isolates in the present study and other species of the genus *Badnavirus* available in the GenBank and one species of the genus *Tungrovirus*.

Phylogeny based in nucleotide sequences from domain RT/RNAseH of the badnaviruses ORF3, revealed that isolates in the present study were clustered with the sequence of PBCoV_EU377664 (Figure 2). This analysis showed that all 83 isolates formed a monophyletic branch (Group I) including the species PBCoV, which have a common ancestral with the species PBErV (Figure 2). Subgroup I contains isolates collected from all sampled municipalities, while subgroup II includes the majority of isolates coming from the municipalities of Arapiraca and Coruripe, in the State of Alagoas. Group II includes all other species belonging to the genus *Badnavirus* (Figure 2).

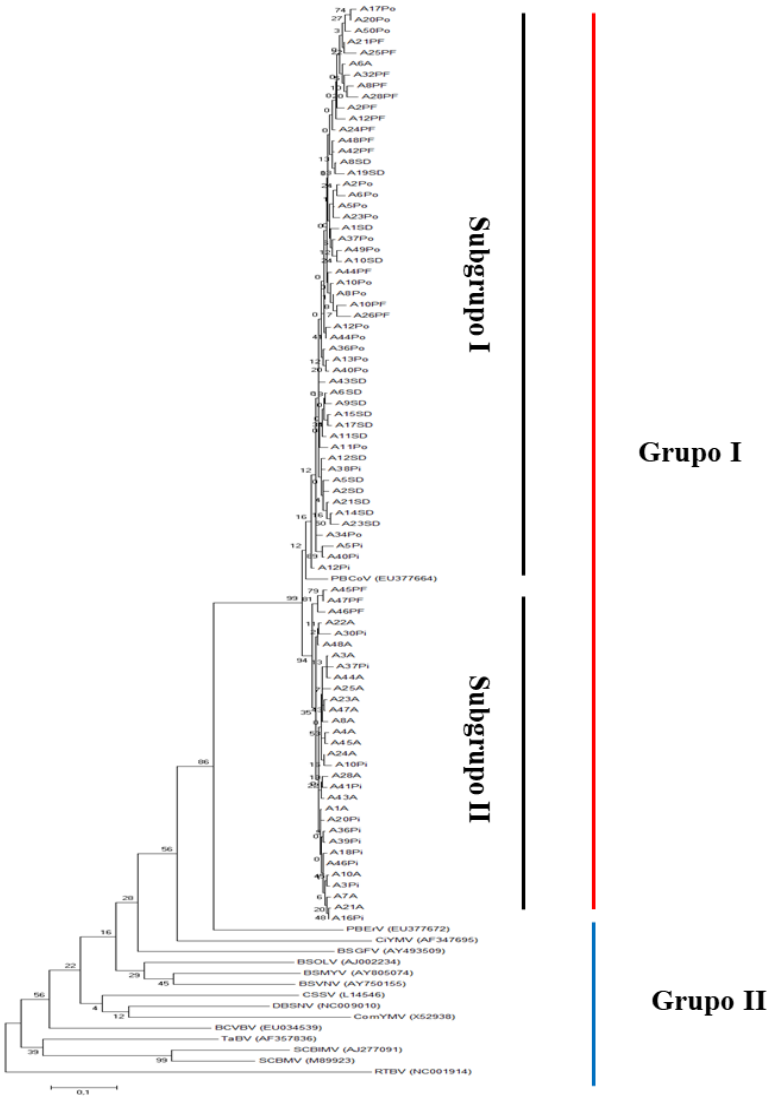

Figure 2. Phylogenetic tree of Maximum Likelihood based in sequences of the RT/RNaseH region of the isolates of *Pineapple bacilliform CO virus* obtained in the present study and other species of *Badnavirus* available in the GenBank. *Rice tungro bacilliform virus* (RTBV) was used as *outgroup*.

The geographic proximity between the municipality of Arapiraca and the village of Pindorama (Coruripe), separated by only 66 km, is the probable reason for the gathering of isolates, once such circumstance fosters the exchange of seedlings between farmers in the region. Paired analysis integrally supports the results obtained by phylogeny, that is to say, groups I in the phylogenetic tree includes only isolates obtained in the present work with the sequence PBCoV, from Australia, which share more than 80% of nucleotide sequence identity in the paired analysis, a decisive criteria to determine species form the genus *Badnavirus*, according to ICTV (GEERING & HULL, 2012).

Thomson et al. (1996) used the PCR technique to detect *Pineapple bacilliform virus* (PBV) in pineapple extracts in Australia and were able to verify the presence of the viral species in all planted areas, constituting a new species of the genus *Badnavirus*. In 2012, using the same approach, a probable new species of *Badnavirus* designated as PBCoV-HI1 and nine variations (A-H) from PBCoV were verified in Hawaii (SETHER et al., 2012). Hernandez-Rodriguez et al. (2013) reported the first occurrence of PBCoV and *endogenous Pineapple pararetrovirus*-1 (ePPRV-1) in pineapple 'cv. Red Spanish' in Cuba. The endogenous form of the virus exerts important epidemiological roll in many cases, once it may originate episomal viruses and consequently elicit the infectious process (DALLOT et al., 2001; DAHAL et al., 2000). Some stress conditions such as tissue culture, hybridization and recombinant events which occur in endogenous sequences, may lead to the reconstitution of the viral genome in the activated form, thus, resulting in episomal infections (CÔTE et al., 2010; DALLOT et al., 2001; NDOWORA et al., 1999). All sequences used in the present work were different from each other. This high variability shows these are probably episomal sequences, once all the EPRVs described until present moment have a similar arrangement pattern with repetitions in *tandem*, internal duplications, fragmentation and inversion of viral genome (GAYRAL & ISKRA-CARUANA, 2009).

Badnavirus are not always detected in symptomatic plants and may also be detected in asymptomatic plants (GAUHL et al., 1997). The absence of symptoms in infected pineapple plants prevents the selection of healthy seedlings for commercialization and planting, resulting in higher dissemination/introduction of the virus in new cropping areas. In addition, symptoms caused by *Badnavirus* in pineapple are not clearly determined (GAMBLEY et al., 2008b; SETHER et al., 2012).

Transmission of PBCoV by mealybugs was verified in Australia with an incidence of 20% and 10% for *D. brevipes* and *P. citri*, respectively (GAMBLEY et al., 2008b). SETHER et al. (2012) observed high incidence of PBCoV (80%) after contact with the mealybug *D. neobrevipes* in Hawaii. The occurrence of *D. brevipes* is verified in all countries where pineapple is cultivated as well as in all Brazilian states (LACERDA et al., 2009). The species *P. citri* has a wide geographical distribution, occurring in tropical, subtropical and temperate regions (CORREA et al., 2008) and the species *D. neobrevipes* was already observed in many fruit crops planted in Brazil (COSTA, 2002). However, the transmission rate of *Badnavirus* by mealybugs is generally considered low, with higher dissemination risk caused by the use of infected propagative material (LOCKHART & OLSZEWSKI, 1993). Therefore, it is believed that pineapple vegetative propagation is one of the main factors contributing to the prevalence of PBCoV in the Brazilian Northeastern region.

CONCLUSIONS

This is the first record of *Badnavirus* infection in pineapple in Brazil. The presence of PBCoV in the states of Alagoas, Maranhão, Paraiba and Pernambuco, suggests a wide distribution and prevalence of this species in the Brazilian Northeastern region, due to the geographic proximity of the cultivation areas evaluated and the absence of phytosanitary barriers, allowing the common practice of exchanging infected seedlings between farmers in cropping areas within these Brazilian states.

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