Detection of *Badnavirus* in pineapple in northeastern Brazil

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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. Análises filogenéticas realizadas revelaram que todas as sequências obtidas neste trabalho apresentaram identidade superior a 80% com a sequência do 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, Paraíba 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 90% 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-CARANNA, 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 (SEETHER & 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).
DETECTION OF Badnavirus IN PINEAPPLE IN NORTHEASTERN BRAZIL
DETECÇÃO DE Badnavirus EM ABACAXIZEIRO NO NORDESTE DO BRASIL

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

<table>
<thead>
<tr>
<th>Collection areas</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Cultivar</th>
<th>No. of samples collected</th>
<th>Sample code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arapiraca (AL)</td>
<td>09°45’09”</td>
<td>36°39’40”</td>
<td>‘Jupi’</td>
<td>60</td>
<td>A1A a A60A</td>
</tr>
<tr>
<td>Pedras de Fogo (PB)</td>
<td>07°24’07”</td>
<td>35°06’59”</td>
<td>‘Pérola’</td>
<td>60</td>
<td>A1PF a A60PF</td>
</tr>
<tr>
<td>São Domingos do Maranhão (MA)</td>
<td>05°34’33”</td>
<td>43°23’07”</td>
<td>‘Pérola’</td>
<td>56</td>
<td>A1SD a A56SD</td>
</tr>
<tr>
<td>Coruripe* (AL)</td>
<td>10°07’32”</td>
<td>36°10’32”</td>
<td>‘Pérola’</td>
<td>51</td>
<td>A1Pi a A51Pi</td>
</tr>
<tr>
<td>Pombos (PE)</td>
<td>08°08’29”</td>
<td>35°29’43”</td>
<td>‘Pérola’</td>
<td>60</td>
<td>A1Po a A60Po</td>
</tr>
</tbody>
</table>

* 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 MgCl₂ 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 BLASTn (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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acronym</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banana streak GF virus</td>
<td>BSGFV</td>
<td>AY493509</td>
</tr>
<tr>
<td>Banana streak OL virus</td>
<td>BSOLV</td>
<td>AJ002234</td>
</tr>
<tr>
<td>Banana streak MY virus</td>
<td>BSMYV</td>
<td>AY805074</td>
</tr>
<tr>
<td>Banana streak VN virus</td>
<td>BSVNV</td>
<td>AY750155</td>
</tr>
<tr>
<td>Bougainvillea chlorotic vein banding virus</td>
<td>BCVBV</td>
<td>EU034559 (=NC011592)</td>
</tr>
<tr>
<td>Cacao swollen shoot virus</td>
<td>CSSV</td>
<td>L14546 (=NC001574)</td>
</tr>
<tr>
<td>Citrus yellow mosaic virus</td>
<td>CYMV</td>
<td>AF347695 (=NC003382)</td>
</tr>
<tr>
<td>Commelina yellow mottle virus</td>
<td>ComYMV</td>
<td>X52938 (=NC001343)</td>
</tr>
<tr>
<td>Dioscorea bacilliform SN virus</td>
<td>DBSNV</td>
<td>DQ822073 (=NC009010)</td>
</tr>
<tr>
<td>Pineapple bacilliform CO virus</td>
<td>PBCoV</td>
<td>EU377664</td>
</tr>
<tr>
<td>Pineapple bacilliform ER virus</td>
<td>PBERV</td>
<td>EU377672</td>
</tr>
<tr>
<td>Sugarcane bacilliform IM virus</td>
<td>SCBIMV</td>
<td>AJ277091 (=NC003031)</td>
</tr>
<tr>
<td>Sugarcane bacilliform MO virus</td>
<td>SCBMOV</td>
<td>M89923 (=NC008017)</td>
</tr>
<tr>
<td>Taro bacilliform virus</td>
<td>TaBV</td>
<td>AF357836 (=NC_004450)</td>
</tr>
<tr>
<td>* Rice tungro bacilliform virus*</td>
<td>RTBV</td>
<td>NC001914</td>
</tr>
</tbody>
</table>

* 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 bp). From 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 of them. Preliminary analysis using the algorithm BLASTn 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, Paraíba 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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