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Identification of cassava bacterial blight-causing *Xanthomonas axonopodis* pv. *manihotis* based on *rpoD* and *gyrB* genes

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Abstract:

Cassava (*Manihot esculenta* Crantz) is one of the most important crops in Vietnam for providing food, starch sources, and raw materials for the production of bio-ethanol and other purposes. Cassava bacterial blight (CBB) disease, caused by *Xanthonomas axonopodis* pv. *manihotis* (*Xam*), is one of the most important factors affecting cassava production in Vietnam. A rapid and sensitive molecular tool is required to support the traditional method, primarily based on biochemical reactions, that was considered less sensitive and time-consuming. In the present study, in order to detect *Xam*, the Polymerase Chain Reaction (PCR) technique was applied with the two primer pairs, rpoD_17F/rpoD_1005R and XgyrconpcrF1/Xgyrconrpcr1, to amplify partial sequences of *rpoD* and *gyrB* genes, respectively. The primers directed the amplification of about 900 bp DNA fragments from CBB-infected leaf and stem tissues. All the amplified DNA sequences were identical in each gene and to the *Xam* reference strains (accession numbers KP265376 and KP265378 for the *rpoD* gene, and KP265387 and KP265388 for the *gyrB* gene) from GenBank. The representative sequences of *rpoD* and *gyrB* genes were deposited in GenBank with accession numbers MF774491 and MF774490, respectively. DNA and phylogenetic analysis based on the sequences of *rpoD* and *gyrB* genes have confirmed that *Xam* is the causal agent of CBB in Dong Nai, Vietnam. This is the first confirmed identification of the causal pathogen of CBB disease in Vietnam using molecular tools, and this method is a reliable tool for the detection and identification of other plant bacterial pathogens.

Keywords: **cassava bacterial blight, *gyrB*, PCR, *rpoD*, *Xanthomonas axonopodis* pv. *manihotis*.**

Classification number: **3.1**

Introduction

Cassava (*Manihot esculenta* Crantz) has high starch content, with approximately 30-60% dry matter, and has become one of the most important crops in the world that is both a food source as well as providing materials for a variety of industries. In Vietnam, cassava is a cash crop that provides food, in particular, and material for the production of bio-ethanol and other products. The most important characteristic of cassava is its drought tolerance and it is therefore widely grown in mountainous areas, in marginal soil types, and with non-irrigation systems with low fertiliser requirements. However, in recent years, cassava production in Vietnam has suffered from a number of diseases and insect pests, especially Cassava pink mealybug (*Phenacoccus manihoti*), cassava witches' broom (CaWB), cassava anthracnose, cassava bacterial blight (CBB) diseases, and a new invasive disease called Sri Lankan cassava mosaic virus (SLCMV). On the basis of observations in the field, CBB is considered to be the third important disease of cassava in Vietnam, after CaWB and SLCMV. However, no study of it has been conducted in Vietnam until now. CBB disease caused by *Xam* is one of the important diseases of cassava in Africa and South America, affecting cassava plants from the planting period to harvesting, and reducing cassava production by up to 100% [1, 2].

Traditional methods for the detection and identification

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of bacterial plant pathogens use selected media, analysis of bacterial colonies on agar plate, biochemical tests, pathogenicity tests, and the analysis of fatty acids [3, 4]; however, these methods are usually labour-intensive, time-consuming, and insufficient. The enzyme-linked immunosorbent assay (ELISA) and monoclonal antibodies have also been employed for the characterisation of bacterial pathogens [5]; however, they are no longer widely used because of their low specificity to the pathogens and because they cause cross-reactions.

In recent years, molecular approaches have become the best methods and are widely applied in the detection and identification of plant bacterial pathogens because they are highly specific, quick, and reliable [6, 7]. PCR and DNA analysis, including the Polymerase Chain Reaction (PCR), Amplified fragment length polymorphism (AFLP) [8], nested-PCR, dot-blot assays [9] and Repetitive elements based PCR (rep-PCR) [10], have been successfully used to detect and identify *Xam*.

To date, no information is available on the phenotypic or genetic variability of *Xam* populations in cassava-growing regions in Vietnam. In particular, there are no reports of the use of molecular techniques for detecting and identifying *Xam* that causes CBB disease in Vietnam. In this study, we focus on the application of a PCR technique using specific primer pairs for *rpoD* (RNA polymerase, sigma 70 (sigma D) factor) and *gyrase B* (*gyrB*) genes in combination with phylogenetic analysis to identify *Xam* from cassava plants with symptoms of CBB collected in Dong Nai province.

Materials and methods

Plant materials

Cassava stems and leaves from five plants showing typical symptoms of CBB were collected from CBB-infected fields in Dong Nai province. One healthy cassava plant was collected from the north of Vietnam as a negative control.

Isolation of bacteria and genomic DNA extraction

Isolation of *Xam* from CBB-infected leaves and stems was undertaken as described by [11] and [12], with some modifications as described below. Small pieces of leaf and stem tissue were excised at the border of the healthy and infected tissues. The tissues were then homogenised in 1 ml of sterilised distilled water and incubated for 25 minutes at room temperature. 100 µl of the resulting solution was streaked in LPG medium (5 g glucose, 5 g yeast extract, 5 g peptone, and 15 g agar in a litre of distilled water). Freshly grown single colonies were used for bacterial cultures in 5 ml of liquid LPG medium at 30^{o}C for 12 hrs and shaken at 150 rpm. 3 ml of the resulting bacterial cultures were subjected to genomic DNA isolation using a DNeasy® mini kit (QIAGEN), following the instructions of the manufacturer.

PCR assays

The two primer pairs were used for PCR amplification. The first primer pair corresponded to the *rpoD* gene, rpoD_17F (5'-ATCTGACCTACGCCGAAGTC-3) and rpoD_1005R (5'-CTGCTGCTCGGAGATGATCT-3') [13]; and the second primer pair was alternatively conserved PCR primers corresponding to the *gyrB* gene, XgyrconpcrF1 (5'-AAGAGCGAGCTGTATCTGAAGGACGA-3') and Xgyrconrpcr1 (5'-CGCGTCCTCGATGCGCACCTGCA-3') [14]. DNA was quantified by means of a UV-Vis Spectrophotometer Optima SP-*3000nano* (Indonesia) and diluted to 20 ng/µl. The PCR amplifications were conducted in 25 µl reaction mixtures containing 2.5 µl of 10×PCR buffer (including 15 mM $MgCl_2$), 0.75 µl of each primer (10 µM), 2 µl of dNTP mix (2.5 mM each), 0.125 µl of Taq polymerase, 1 µl of DNA (20 ng/µl), and up to 25 µl of H_2O. The PCR temperature conditions were 4 min at 94^{o}C followed by 35 cycles of 94^{o}C for 30 s, Tm (50^{o}C for 45 s for the XgyrconpcrF1/Xgyrconrpcr1 primer pair, and 60^{o}C for 30 s for the rpoD_17F/rpoD_1005R primer pair), and 72^{o}C for 1 min, with a final extension at 72^{o}C in a Mastercycler Pro (Eppendorf, Germany). The PCR products were separated by electrophoresis in 1% agarose gel in a 1xTAE buffer containing 0.5 µg/ml ethidium bromide and photographed under UV light using a GelDoc-It® 310 Imaging System (United Kingdom).

Phylogenetic analysis

The target PCR fragments were cut out and purified using a QIAquick Gel Extraction kit (QIAGEN, Cat No./ ID: 28704) and directly sequenced with both forward and reverse primers that were used in PCR assays with ABI3100 sequencer. The DNA sequences were subjected to the National Centre for Biotechnology Information BLAST search tool [15] to ascertain the closest match. The multiple DNA sequences were aligned with the clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic trees were constructed using MEGA 7.0 [13] by means of the neighbour-joining method with default values and 1,000 bootstrap replications.

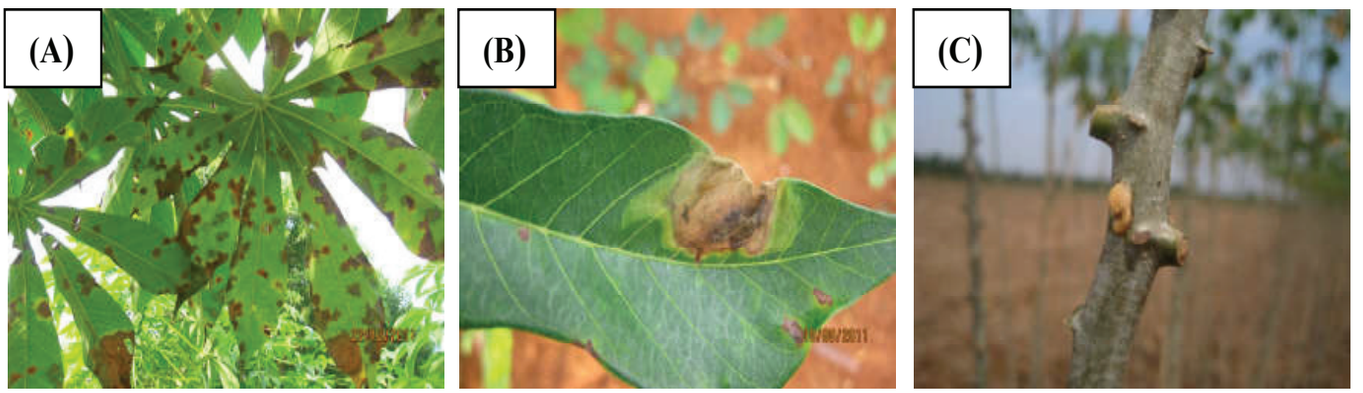

Fig. 1. Typical symptoms of CBB disease on cassava plants grown in Dong Nai province, Vietnam. (**A, B**) symptoms on leaves and (**C**) on stem.

Results and discussion

The symptoms of CBB disease

The typical symptoms of CBB disease in cassava caused by *Xam* include blighting, wilting, die-back of stems, formation of small angular leaf spots (Fig. 1A) that can become larger (Fig. 1B) and surrounded by yellow edge, vascular necrosis of the stem, and the production of exudates on the leaves and stem (Fig. 1C).

Detection and phylogenetic analysis of Xam based on partial rpoD gene sequences

PCR products with an expected length about 900 bp were amplified from two leaf samples and one stem sample by the rpoD_17F/rpoD_1005R primer pair. No amplicon was detected in the negative control (a healthy cassava plant) (Fig. 2). All three PCR products were purified with agarose gel and directly sequenced both directions using rpoD_17F and rpoD_1005 primers. Sequencing results showed that DNA sequences from the three isolates were identical. BLAST search results indicated that strains were 100% identical to strains of *Xam* (accession numbers: KP265372, FJ561609, and FJ561607). The CBB isolated from Dong Nai province that used *rpoD* as the target gene was designated CBBDNVN-*rpoD* and deposited in GenBank with the accession number MF774491.

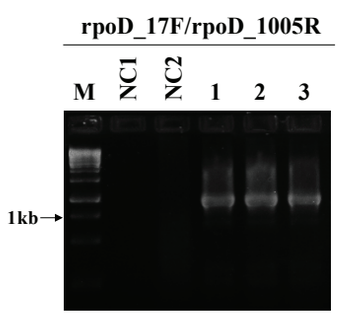

Fig. 2. PCR using primer pair rpo_17F and rpoD_1005R for amplification of the *rpoD* gene of *Xam* isolated from the CBB-infected samples collected from Dong Nai province. M is the DM3100 DNA ladder (SMOBIO), NC1 is DNA extracted from a healthy cassava plant, NC2 is no DNA, 1-3 are two leaf isolates and one stem isolate from the CBB-infected leaf samples and stem sample, respectively, collected from Dong Nai province.

The phylogenetic tree was constructed with nucleotide sequences of CBBDNVN-*rpoD* and 21 other *rpoD* sequences of *Xanthomonas* species from GenBank with the accession number shown in parentheses in the Fig. 3 and was analyzed by means of the neighbour-joining method with 1,000 bootstrap replications using MEGA7.0 [16]. The most ancestral *Xanthomonas* species within the phylogenetic tree were *X. axonopodis* pv. *manihotis* and *X. axonopodis* pv. *alfalfa*e. The CBBDNVN-*rpoD* shares 100% homology with the *X. axonopodis* pv. *manihotis* and is therefore considered to be among the most ancestral species in this phylogenetic tree, confirming that it belongs to the pathovar *manihotis*. The remaining *Xanthomonas* species were separated into three major groups. Group I, supported by bootstrap values of 81%, comprised *X. translucens*, *X. oryzae*, *X. sacchari*, *X. theicola*, *X. albilineans*, and *X. hyacinthi*. Group II, supported by bootstrap values of 100%, comprised *X. vesicatoria*, *X. cassavae*, *X. dyei*, *X. vasicola*, *X. pisi*, and *X. bromi*. Group III, also supported by bootstrap values of 100%, comprised *X. melonis*, *X. fragariae*, *X. cynarae*, *X. cucurbitae*, *X. codiae*, and *X. alfalfae* subsp. *Citrumelonis* (Fig. 3).

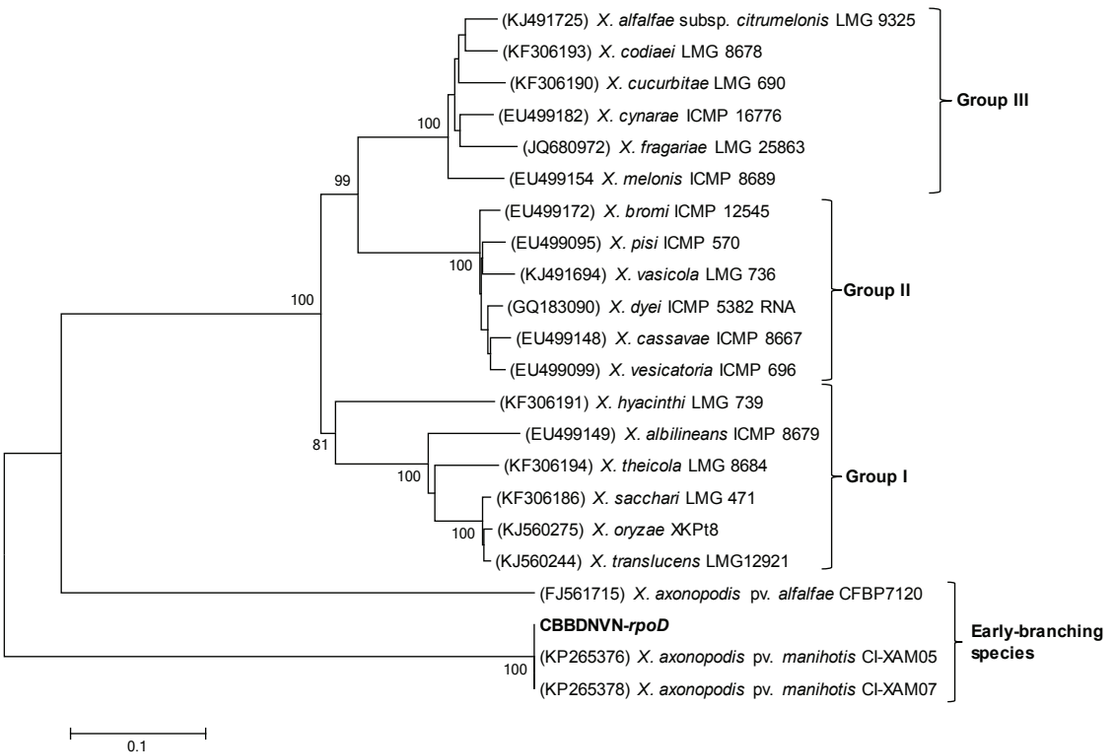

Fig. 3. Phylogenetic tree constructed by the neighbour-joining method, comparing the CBBDNVN-*rpoD* sequence with 21 other *rpoD* gene sequences of different *Xanthomonas* species from GenBank. Accession numbers are shown in parentheses. The numbers at the branches are confidence percentages obtained from 1,000 bootstrap replicates (only values above 80% are shown).

Detection and phylogenetic analysis of Xam based on partial gyrB gene sequences

Primer pair XgyrconpcrF1/Xgyrconrpcr1 directed the amplification of about 900 bp of the target DNA sequence of the *gyrB* gene from two CBB-infected leaf samples and from one CBB-infected stem sample (Fig. 4).

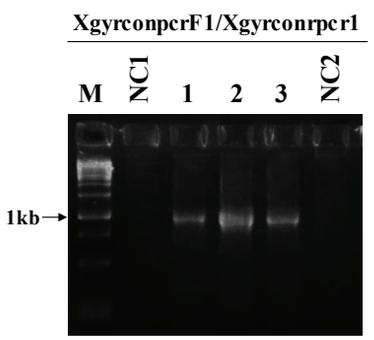

Fig. 4. PCR using primer pair XgyrconpcrF1/Xgyrconrpcr1 for amplification of the *gyrB* gene from bacteria isolated from the CBB-infected samples collected from Dong Nai province. M is the DM3100 DNA ladder (SMOBIO), NC1 is DNA extracted from a healthy cassava plant, NC2 is no DNA, 1-3 are two leaf isolates and one stem isolate from the CBB-infected leaf samples and stem sample, respectively, collected from Dong Nai province.

The CBB isolated from Dong Nai province that used *gyrB* as the target gene was designated CBBDNVN-*gyrB*. This shares 100% homology with *X. axonopodis* pv. *manihotis* CI-XAM04 (accession number KP265387) and *X. axonopodis* pv. *manihotis* CI-XAM05 (accession number KP265288) which are the pathovars that cause CBB. They are early-branching species in the phylogram. The phylogenetic analysis confirms that CBBDNVN-*gyrB* (deposited in GenBank with the accession number MF774490) belongs to the pathovar *manihotis* and is the causal agent of CBB in Vietnam.

Difference in comparison with the phylogenetic tree based on the sequences of the *rpoD* gene, the remaining *Xanthomonas* species were separated into three major groups. Group I, supported by bootstrap values of 100%, comprised *X. hyacinthi*, *X. translucens*, *X. theicola,* X. *albilineans*, and *X. sacchari*. Group II, supported by bootstrap values of 99%, comprised *X. cassavae*, *X. codiaei*, and *X. cucurbitae*. Group III comprised a large group and was divided into three clades IIIA, IIIB, and IIIC. Clade IIIA, supported by bootstrap values of 86%, comprised *X. vesicatoria* and *X. pisi*; clade IIIB comprised *X. cynarae*, *X. populi*, and *X. fragariae*; and clade IIIC, supported by bootstrap value of 100%, comprised *X. oryzae*, *X. vasicola*, *X. bromi*, *X. alfalfae*, *X. axonopodis*, and *X. melonis* (Fig. 5).

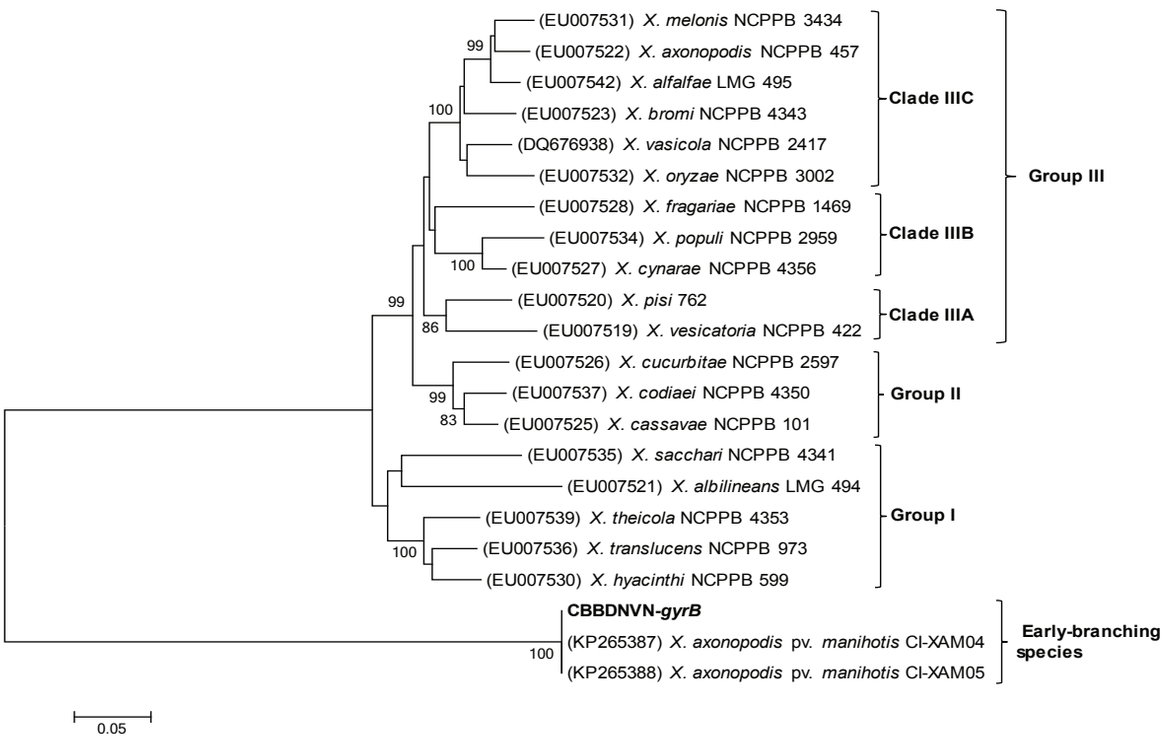

Fig. 5. Phylogenetic tree constructed by the neighbour-joining method, comparing the CBBDNVN-*gyrB* sequence with 21 other *gyrB* gene sequences of different *Xanthomonas* species from GenBank. Accession numbers are shown in parentheses. The numbers at the branches are confidence percentages obtained from 1,000 bootstrap replicates (only values above 80% are shown).

Xanthomona comprises a large group of bacterial species with various physiological and pathological characteristics; they are causal agents of different diseases of many important crops. In recent years, molecular approaches using different target genes have been applied to distinguish between species within *Xanthomonas*. [11] developed a PCR method with specific primer pairs XV (5′-TTC-GGC-AACGGC-AGT-GAC-CAC-C-3′) and XK (5′-TCA-ATC-GGA-GAT-TAC-CTGAGC-G-3′) which proved to be a relatively sensitive method for detecting *Xam* in cassava stem and leaf lesions with the minimum number of cells (3×10^2 CFU/ml) could be detected. A specific, sensitive, quick nested-PCR technique using two primer pairs XK/XV and XV2 (5'-AACAGT-AAG-TCG-GTG-TCG-CC-3')/XK2(5'-TCG-TTG-CCG-TGG-CTA-CC-3') successfully detected *Xam* from cultured cells, artificially inoculated seeds, and naturally infected seeds [9]. The phenotypic and genetic variability of *Xam* isolates was determined using DNA polymerase chain reaction-based fingerprinting (rep-PCR) [10].

Different genes have been used as targets for phylogenetic analysis, such as the 16S rRNA gene [17], the 16S-23S rRNA intergenic spacer region [18], *rpfB* and *atpD* genes [8], the *gyrB* gene [14, 19], and the *rpoB* gene (which encodes the β subunit of bacterial RNA polymerase) [20]. Previous studies indicate that approaches based on the analysis of 16S rDNA sequences and the 16-23S intergenic region revealed very low diversity, with most of the species clustered into a single group [18, 21]; therefore, neither of these target genes would reliable for the differentiation of the majority of *Xanthomonas* species. [14] constructed a phylogram using different *gyrB* sequences and indicate that the *gyrB* gene is a reliable target gene for rapid, accurate identification at the species level among *Xanthomonas* species. The *rpoB* gene, a highly conserved housekeeping gene with a single copy in the genome [22], has also been used as a target gene for the detection, characterisation, and discrimination of several bacterial species, including *Xanthomonas* [20].

The *rpoD* and *gyrB* genes have been used as molecular markers for the rapid detection and characterisation of *Xam* in Burkina Faso [23] and in the Ivory Coast [13], suggesting that these genes can be used for the study of a variety of bacterial pathogens.

In the present study, we applied a PCR technique using the two primer pairs, rpoD_17F/rpoD_1005R and XgyrconpcrF1/Xgyrconrpcr1, and provided evidence that they are appropriate targets for the detection and phylogenetic analysis of *Xam* in the CBB-infected cassava plants. The method is quite specific and quick in detecting *Xam* from

bacterial cultures, suggesting that it is a good method for the detection and characterisation not only of the *Xanthomonas* genus but others too.

Conclusions

The causal agent of CBB disease in samples collected from Dong Nai province, Vietnam, is *Xanthomonas axonopodis* pv. *manihotis*. The PCR technique with primer pairs rpoD_17F/rpoD_1005R and XgyrconpcrF1/Xgyrconrpcr1, involving the amplification of the target *rpoD* and *gyrB* genes, is reliable method for the rapid detection and identification of the *Xam* that causes CBB disease.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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