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Improvement of the specific detection of *Xanthomonas phaseoli* pv. *manihotis* based on the *pthB* gene

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ABSTRACT. Modifications were made in the PCR conditions aiming to overcome the problem of non-amplification of the *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) fragment, using the primer pair XV / XK described in the literature. The objective of this study was to propose changes in the primers already described (XV / XK_MOD) and validate the use of these new primers in identifying *Xpm*. The validation procedure was carried out with the primer pair XV and XK_MOD, using different strains of *Xpm*, other plant pathogenic and endophytic bacteria genera and one isolate of *X. phaseoli* pv. *passiflorae*. As a control, additional reactions were conducted in multiplex with the universal primers for the 16S rRNA gene of the bacteria together with XV / XK and XV / XK_MOD. Using the forward primer (XV) described in the literature together with the modified reverse primer (XK_MOD), it was possible to achieve amplification from DNA extracted from *in vitro* cultures and from infected tissue, but no amplification was noticed for the primer pair described in the literature, confirming the effectiveness of the proposed modification.

Keywords: *Xpm*; cassava bacterial blight; molecular diagnosis; resistance.

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Introduction

Bacterial blight (CBB), caused by the bacteria *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*), formerly named *X.* aonopodis pv. *manihotis* (Constantin et al. 2016) is one of the most destructive diseases of cassava (*Manihot esculenta* Crantz) (Parkes, Fregene, Dixon, Boakye-Peprah, & Labuschange, 2013). CBB is a foliar and vascular disease characterized by angular leaf lesions, blight, wilting, stem exudates, stem cankers, water-soaked lesions, angular necrotic lesions on the leaf blade, and necrotic lesions on the petioles and stem with gummosis (gum exudation) (Lopez & Bernal, 2012; Wonni et al., 2015).

According to Mansfield et al. (2012), the *Xanthomonas axonopodis* (syn= *X. phaseoli*) species is one of the "Top 10" most important plant pathogenic bacteria, with *Xpm* being the most destructive pathovar of the genera.

Cuttings (manivas) are the primary source of inoculum for the next cycles of the crop; since *Xpm* infects the stems and, in most cases, the stems are symptomless, they are taken to regions free of the disease (Lopez & Bernal, 2012) and become a new source of infection. Rapid and accurate detection of the causal agent is an important factor for quarantine and thus prevents the spread of the disease by infected plant material. Classic and routine methods of phytobacterium diagnosis using biochemical, serological and physiological characteristics are time-consuming and are less accurate when compared to the currently employed molecular methods, such as PCR (Sousa et al., 2015). The availability of primers has led to widespread application of molecular techniques for assessing the distribution of existing pathogens, the occurrence of new/introduced pathogens or strains and the prevention of introduction of new pathogens.

Given the great importance of cassava cultivation and losses generated by CBB incited by *Xpm*, specific primers were developed by Verdier, Mosquera, and Assigbétsé (1998) to obtain a rapid diagnosis of pathogens based on a specific region from pathogenic isolates of *Xpm* (*pthB*). Despite the specificity, these primers were not able to amplify the specific fragment corresponding to *Xpm* in the different trials conducted in the Molecular Biology Laboratory at Embrapa Mandioca e Fruticultura, Cruz das Almas, Bahia, State, Brazil.

Thus, this study aimed to create and evaluate a modification in the reverse primer described by Verdier et al. (1998) to (i) allow *Xpm* detection by conventional PCR in infected plant tissue and from pure colonies; (ii) assess their sensitivity and specificity and (iii) assess the possibility of using these primers as an indexation tool. The work also presents some insights into the systemic movement of the bacteria by the enhanced primers and its relationship with the resistance level in cassava varieties.

Material and methods

To design a new set of primers, the same nucleotide sequence (GenBank accession No. AF012325) used by Verdier et al. (1998) was used as a template. The following steps were carried out: search and selection of sequences of interest - the sequence of the *pthB* region used by Verdier et al. (1998) and that same region from different *Xpm* isolates available in GenBank, in addition to the complete genome of some *Xpm* strains. Sequences were aligned, and a consensus sequence was generated using GeneFisher2 software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/); from that sequence, a search of the best combinations was performed to obtain the primers using the Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0).

The different bacterial isolates used in the tests were obtained from the Coleção de Culturas de Fitobactérias do Instituto Biológico (IBSBF) and from the Bacteria collection at Embrapa Mandioca e Fruticultura (CBEMF) (Table 1). The isolates were recovered in YPG culture medium (5 g of yeast extract, 5 g of proteose [peptone], 5 g of glucose, and 15 g agar per litre of distilled water) and incubated at 28°C for 48 hours until DNA extraction (Verdier et al., 1998). The isolates were stored in sterile distilled water (SDW) and in 20% glycerol at -20°C.

The original primer pair and the modified one (XK_MOD) were tested for specificity in PCR detection assays using DNA from 19 bacterial isolates from different genera and *Xanthomonas* pathovars (Table 1).

Table 1. Bacteria species used in specificity tests of primers XV / XK_MOD.

Species	Strains	Origin	Crop
Pseudomonas viridiflava	CBEMF-1[a]	Unknow	Chinese Cabbage
P. syringae pv. *maculicola*	CBEMF-2	Unknow	Cauliflower
Pseudomonas marginalis	CBEMF-3	Unknow	Lettuce
Ralstonia solanacearum	CBEMF-4	Unknow	Cucumber
Xanthomonas campestris	CBEMF-5	Unknow	Sweet Pepper
Bacillus sp.	CBEMF-6	Cruz das Almas – BA	Soil sample
Pectobacterium carotovorum subsp. *carotovorum*	CBEMF-7	Pernambuco (UFRPE)	Garlic
X. axonopodis pv. *passiflora* (Xap)	CBEMF-8	Pernambuco (UFRPE)	Passionfruit
X. phaseoli pv. *manihotis*	CBEMF-9	Brasília – DF	Cassava
Enterobacter aerogenes	CBEMF-19	Guanambi – BA	Cassava Endoph.[b]
Enterobacter cloacae	CBEMF-11	Guanambi – BA	Cassava Endoph.[b]
Enterobacter asburiae	CBEMF-23	Guanambi – BA	Cassava Endoph.[b]
Klebisiela oxycota	CBEMF-105	Dourados– MS	Cassava Endoph.[b]
Kosakonia cowanii	CBEMF-112	Laje – BA	Cassava Endoph.[b]
Klebisiella variicola	CBEMF-128	Marechal C. Rondon – PR	Cassava Endoph.[b]
X. phaseoli pv. *manihotis* (*Xpm*)	IBSBF 278	Unknow	Cassava
X. phaseoli pv. *manihotis*[*]	IBSBF 291	Unknow	Cassava
X. phaseoli pv. *manihotis*[*]	IBSBF 1994	Unknow	Cassava
X. phaseoli pv. *manihotis*[*]	IBSBF 436	Unknow	Cassava
X. phaseoli pv. *manihotis*[*]	IBSBF 328	Unknow	Cassava
X. phaseoli pv. *manihotis*[*]	IBSBF 726	Unknow	Cassava
X. phaseoli pv. m*anihotis*	PR	Naviraí – PR	Cassava

CBEMF: Bacteria collection from the Phytopathology Laboratory at Embrapa Mandioca e Fruticultura; IBSBF: Collection of cultures of Phytobacteria from the Institutlo Biológico. bSpecies found as cassava endophyte associated with bacterial blight lesions Only DNA from the bacteria donated from the Instituto Biológico, SP. Abbreviation: BA: Bahia; MS: Mato Grosso do Sul; PR: Paraná; SP: São Paulo; UFRPE: Universidade Federal Rural de Pernambuco.

The isolates grew for 48 hours at 28°C in test tubes with inclined solid YPG medium. Afterwards, 1 mL of sterile distilled water was added to each tube, and the suspension was transferred to 2 mL microtubes. The suspension was incubated for 10 minutes at 65°C in a water bath and then centrifuged under refrigeration at 21,000 (*g*) for 7 minutes. The supernatant was collected and measured. Quantification was performed by electrophoresis (90 V / 50 min.) in a 1% agarose gel in 0.5X TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.0). The DNA of lambda (λ) phage was used as a marker at concentrations of 50 and 100 ng for comparative analysis of DNA. After estimation of the concentration, samples were diluted to 5 ng μL^{-1}. Total and diluted DNA samples were stored at 4°C.

PCR Reaction

PCR reactions were performed using 30 ng of DNA, 1X Taq buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl), 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 mM primer F (XV: 5'-TTC-GGC-AAC-GGC-AGT-CAG-CAC C-3'), 0.2 mM R primer (proposed here) (XK_MOD: 3'-CGG-AGA-AAT-TTA-CCT- GAG CG-5'), and 0.3 U of Taq DNA polymerase in a final reaction volume of 12.5 µL. PCR was performed under the following conditions: initial denaturation at 95°C for 5 min.; followed by 30 cycles of denaturation for 30 s at 95°C, primer annealing at 61°C for 30 s, and extension at 72°C for 1.5 min.; ending with one cycle of 7 min. at 72°C. The reaction product of approximately 900 bp was analysed by agarose gel electrophoresis in 1% (80 V / 90 min.) in 0.5X TAE buffer (Verdier et al., 1998, adapted).

Multiplex PCR Reaction

Simultaneous amplification tests were performed with universal primers for bacteria 16S8F (5'-AGA-GTT-TGA-CTC-TGG-TCM-AG-3') / 16S1492R (3'-TAC CTT -GTT-GCA T-TCA-5') (Turner, Pryer, Miao, & Palmer, 1999), and specific primers Xpm XV / XK (XV: 5'-TTC-AAC-GGC-GGC-AGT-CAG-CCC-C 3' and XK: 3'-TCA-ATC-GGA-GAT-tac-CTG-AGC-G-5') and the proposed modification XV / XK_MOD (XV: 5'-TTC-GGC-AAC-GGC-AGT-GAC-CAC-C 3' and XK_MOD: 3'-AAT-CGG-AGA-TTA-CCT-GAG-CG-5'). The universal primers for bacteria were used to ensure that the PCR reactions occurred.

PCR reactions for amplification of DNA with Xpm 16S8F / 16S1492R primers; XV / XK and 16S8F / 16S1492R; XV / XK_MOD 16S8F and / 16S1492R were performed with 30 ng of DNA, 1X Taq buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl), 2.5 mM MgCl2, 0.2 mM dNTPs, 0.33 mM F primer, 0.33 mM of the R primer, and 1 U of Taq DNA polymerase, with a final reaction volume of 15 µL. PCR was performed under the following conditions: initial denaturation at 95°C for 4 min.; followed by 40 cycles of denaturation for 30 s at 94°C, primer annealing at 51°C for 30 s, and extension at 72°C for 1.5 min.; ending with one cycle of 7 min. at 72°C. The reaction product of amplification from 1,000 to 1,200 bp was analysed by electrophoresis on a 1% agarose gel (80 V / 90 min.) in 0.5X TAE buffer (Verdier et al., 1998, adapted).

The sensitivity of the 4 primers was tested in the evaluation of the detection limit. Total DNA isolated from IBSBF 278 (Table 1) was used at concentrations of 100, 75, 50, 25, 1, and 0.25 ng to test the limit of detection of the selected primer pair in the PCR reaction. The same amplification conditions mentioned above were used with the primers developed by Verdier et al. (1998) to compare the detection capability of both primer pairs.

Xpm detection from infected tissue

Two-month-old plants of four cassava varieties (Cigana Preta, BRS Kiriris, Salangor and BRS Verdinha), differing from each other in the level of quantitative resistance, were inoculated by the toothpick method, as described by Muñoz-Bodnar et al. (2014), with the isolate IBSBF 278, and maintained under controlled conditions (28°C with a photoperiod of 12h) in a growth chamber for the development of symptoms. After 15 days of inoculation, the stem and leaf tissues with symptoms were collected, and total DNA was extracted.

Total DNA extraction was performed using the Doyle and Doyle (1987) protocol with modifications. Briefly, 300 mg of each collected tissue was macerated and homogenized using a mortar and pestle with liquid nitrogen. The macerate was transferred to a 2 mL microtube, and 1 mL of 2% CTAB extraction buffer was added. Afterwards, the samples were incubated at 65°C for 45 min. and homogenized every 15 min. They were then incubated at -20°C for 5 min., and 600 µL of chloroform: isoamyl alcohol (24:1) was added, followed by rapid vortexing and centrifugation for 10 min. at 13,000 (g). Thereafter, 800 µL of the supernatant was collected into a new microtube to which 600 µL of isopropanol was added and incubated at -20°C for at least 1 hour to precipitate the DNA.

The samples were centrifuged at 13,000 g for 10 min., and the supernatant was discarded to obtain the DNA pellet. Each DNA pellet was washed with 500 µL of 70% ethanol and centrifuged at 13,000 g for 5 min. The supernatant was discarded, and traces of ethanol were removed by drying the pellet for 15 min. at 37°C. The pellet was resuspended in 100 µL of TE buffer (10 mM Tris-HCL + 1 mM EDTA) treated with 1% RNase and incubated for 1 hour at 37°C. After incubation, 10 µL of sodium acetate was added (3 M), and after homogenization, 200 µL of absolute ethanol was added, and the samples were incubated for 1 hour at -20°C for DNA precipitation

After incubation of the DNA, a new centrifugation for 10 min. at 13,000 g was performed, and the supernatant was discarded. Then, 1 mL of 70% ethanol was added, and the samples were left to air dry at room temperature for 10 min. and centrifuged at 14,000 g for 5 min. to obtain the pellet. The supernatant was discarded, and the pellet was dried for 20 min. at 37°C. The DNA was resuspended in 70 µL of sterile TE buffer. DNA quantification was performed in a 1% agarose gel using TAE buffer and stained with ethidium bromide (0.5 µg mL^{-1}). A total of 30 ng of DNA was used for the PCR reactions.

Detection in different portions of the stem

To assess the application of primers in studies of the systemic movement of the bacteria on the host plant and its correlation with the level of resistance of the varieties, an experiment in the growth chamber was conducted using a completely randomized design (CRD) (9 cassava genotypes x 1 *Xpm* isolate x 7 replicates). Two-month-old cuttings of the commercial varieties, BRS Caipira, BRS Kiriris, BRS Poti Branca, BRS Tapioqueira, BRS Verdinha, Cigana Preta, Salangor, and two hybrids, 98150-06 and 9624-09, developed by the cassava breeding programme at Embrapa Cassava and Fruits, were housed in a moist chamber for 24 hours before inoculation. Inoculation was performed with a sterilized toothpick at a point near the base of the stem, according to the methodology proposed by Muñoz-Bodnar et al. (2014). As a control, cuttings of each variety were inoculated with a toothpick soaked in sterile distilled water.

After fifteen days of inoculation, two plants from each variety were used in the detection test at different portions of the stem: (i) 3 cm below the point of inoculation (base of the stem next to the substrate); (ii) the exact point of inoculation; (iii) 8 cm above the inoculation point and (iv) 16 cm above the inoculation point.

The total DNA of the samples was extracted as cited above according to the modified Doyle and Doyle (1987) protocol, and the PCR reactions were performed as described previously for the XV / XK primer pair and the reverse modification XK_MOD.

Results and discussion

The pair of primers for molecular detection by conventional PCR of *Xpm* chosen for this study and validated after *in silico* tests was 5'- TTCGGCAACGGCAGTGACCACC - 3' (XV) (Verdier et al., 1998) and 3'-AATCGGAGATTACCTGAGCG - 5'(XK_MOD) (proposed in this study). 'XK_MOD' differs from 'XK' as described by Verdier et al. (1998) in size; 'XK-MOD' contains 20 nucleotides (excluding the first two nitrogenous bases [TC]) and has a Tm of 52°C (the original has a Tm of 55°C), amplifying a specific ~900 base pair (bp) fragment.

Different regions of the bacterial genome have been used to develop primers for bacteria of the Xanthomonas genus, such as RAPD, AFLP, VNTR, and SSR markers (Trujillo et al., 2014; Arrieta-Ortiz et al., 2013; Ogunjobi, Fagade, & Dixon, 2010). However, the hrp and pthB regions, which are fairly conserved within this group, are used and provide the most accurate results when diagnosing different species and pathovars (Al-Saadi et al., 2007). The knowledge of this specificity helps in choosing the optimum region for primer design since they exclude the possibility of amplification of different species and can accurately target the region of specific pathovars, as was shown by Verdier et al. (1998), González, Restrepo, Tohme, and Verdier (2002) and in this work.

Within the possible options generated by the Primer3 analysis, the regions that introduce the fewest changes in the reaction were chosen. The reverse primer proposed and validated here (XK_MOD) is a modification of 'XK' designed by Verdier et al. (1998), differing in the exclusion of the initial two bases (TC) in the 5' position. Despite the reduction in the number of bases, the primer had an ideal size because XK contained 22 bp that included the 20 nitrogenous bases of XK_MOD (Wensing, Gernold, & Geider, 2011).

There was no difference in the possibility of formation of secondary structures (hairpin) and primer dimer formation by *in silico* analysis between the two primers. However, the presence of polymorphism at the two bases of the 5' region of the XK primer observed in the analysed sequences, along with the formation of secondary structures, are probably the factors that prevented amplification of different sequences used by Verdier et al. (1998). This factor (polymorphism in the region of the first two bases of the primer XK) can result in a "repulsion" between the primer and the template strand since the bases are not complementary, preventing perfect primer annealing, which leads to inaccurate annealing, preventing amplification.

Specificity of the primers

The specificity of the primers proposed in the literature and the modification was tested by conventional PCR using the different bacteria species and *Xanthomonas* pathovars listed in Table 1. As expected, there was only the amplification of a DNA fragment of approximately 900 bp in the *Xpm* isolates used in the reactions (Figure 1).

The amplification of a fragment specific to the *Xpm* isolate with the primer pair XV / XK_MOD showed a high degree of specificity of these primers and their ability to detect the pathogen with high reliability. Hence, the primer pair designed and validated in this study came from a modification of the primers designed by Verdier et al. (1998); their specific nature was previously known, and for instance, had already been proven. This is because the authors used 112 *Xpm* isolates (107 pathogenic and five nonpathogenic)

from 25 countries in South America, Asia and Africa, as well as 46 other samples of bacteria, including other species of *Xanthomonas*, different pathovars of *Xanthomonas axonopodis* and species from other genera, including epiphytes isolated from cassava leaves with CBB symptoms.

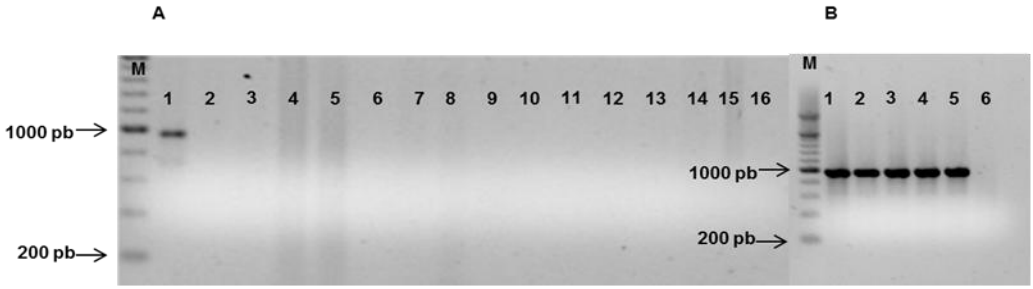

Figure 1. Specificity test of the primers XV / XK_MOD, with bacteria isolates from different genera and species. (A):1: *Xanthomonas phaeoli* pv. *manihotis* (IBSBF 278); 2: *X. axonopodis* pv. *passiflorae* (CBEMF-8); 3: *Xanthomonas campestris* (CBEMF-5); 4: *Ralstonia solanacearum* (CBEMF-4); 5: *Pseudomonas marginalis* (CBEMF-3); 6: *Pseudomonas syringae* pv. *maculicola* (CBEMF-2); 7: *Pectobacterium carotovorum* subsp. *carotovorum* (CBEMF-7); 8: *Pseudomonas viridiflava* (CBEMF-1); 9: *Enterobacter aerogenes* (CBEMF-19); 10: *Enterobacter cloacae* (CBEMF-11); 11: *Enterobacter asburiae* (CBEMF-23); 12: *Klebsiella oxytoca* (CBEMF-105); 13: *Kosakonia cowanii* (CBEMF-112); 14: *Klebsiella variicola* (CBEMF-128); 15: *Bacillus* sp. (CBEMF-6); 16: control (without DNA). (B): Isolates of *X. phaeoli* pv. *manihotis*. 1: IBSBF 278; 2: IBSBF 1998; 3: IBSBF 291; 4: IBSBF 436, 5: CBEMF-9; and 6: control (without DNA). M: 200 bp Ladder (Invitrogen).

Sensitivity of the primers

The sensitivity of the primers was assessed using six concentrations of DNA *Xpm* (IBSBF 278) for detecting the lowest detection limit to compare with the pair of primers developed by Verdier et al. (1998). For the modified primer, six concentrations were used (100, 75, 50, 25, 1, and 0.25 ng) with three replicates (Figure 2A). For the reactions using the pair of primers developed by Verdier et al. (1998), there was no amplification in the concentrations used (Figure 2B). The sensitivity of detection can be considered the efficiency of identifying and diagnosing the pathogen of interest, without the formation of false positives and negatives that can be caused by cross-reactions, reactions performed poorly or poor development of diagnostic protocols.

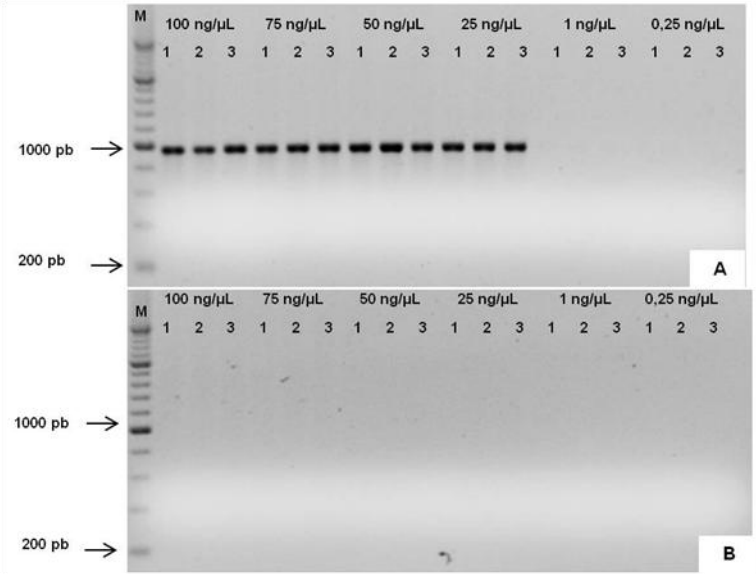

Figure 2. PCR reaction in 1% agarose gel electrophoresis using DNA from the IBSBF 278 (*Xanthomonas phaeoli* pv. *manihotis*) isolate in different concentrations, in the following order: 100, 75, 50, 25, 1, and 0.25 ng with three replicates each (1, 2, and 3). A: Reaction with primers XV/XK_MOD; B: Reaction with primers XV/XK (Verdier et al., 1998). M: 200 bp DNA ladder.

The lower limit of detection of target DNA corresponds to the smallest value (pathogen) that can possibly be detected in a sample (Teló, Machado, Schmitt, & Chesky, 2007) and is important for the standardization of protocols used in different laboratories. The lower limit of detection observed in this study differs from those observed in the work of Simenc and Potocnik (2011) and Trindade, Marques, Lopes,

and Ferreira (2007), where amplifications were possible at concentrations of 0.0002, 0.00025, 0.001, and 0.01 ng of purified DNA per reaction. This may be related to the purity of the DNA in the reaction since the purified DNA is free of inhibitors that can interfere with the efficiency of the amplification, unlike the test samples, which are not purified and may limit the power of detection.

Detection from the host tissue by PCR

In the screening test using DNA extracted from cassava leaves and stems with symptoms of bacterial blight, there was positive amplification in all samples using the pair of primers developed in this work (Figure 3).

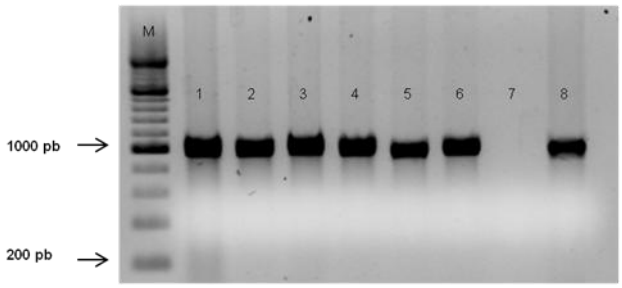

Figure 3. PCR reaction in 1% agarose gel electrophoresis using DNA from the plants inoculated with IBSBF 278 (*Xanthomonas phaeoli* pv. *manihotis*) isolate. 1: BRS Tapioqueira (stem); 2: Cigana Preta (leaf); 3: Cigana Preta (stem); 4: Salangor (leaf); 5: BRS Poti Branca (leaf); 6: BRS Poti Branca (Stem); 7: negative control (plant material not infected with *Xpm*); 8: positive control (*Xpm* IBSBF 278 isolate). M: 200 bp DNA ladder.

The results presented here show that it is possible to diagnose the presence of *Xpm* from infected tissue and that the pathogen can be efficiently detected. The diagnosis of the pathogen directly from infected tissues is a faster and more reliable strategy than the pathogen isolation technique since using traditional approaches can lead to misidentification of the bacterial strains and possibly generation of false positives or negatives, compromising the accuracy of results due to the presence of endophytic and saprophytic bacteria (Miller, Beed, & Harmon, 2009). Therefore, a rapid and specific diagnosis to prevent the spread of the pathogen enables control measures before disease progression in the planting season.

By using the primer pair XV / XK_MOD in symptomatic and asymptomatic tissues in different portions of the stem, it was possible amplify the product in all samples (Figure 4) after 15 days of inoculation. This corroborates the results of Trindade et al. (2007) and Adikini et al. (2011), where it was possible to detect bacteria in infected plant tissue using PCR by amplifying the specific fragment of DNA from the pathogen. The capacity to detect the bacteria in asymptomatic tissues allows the application of primers in the diagnosis of both symptomatic and asymptomatic infected plant tissue since the bacteria may systemically colonize without showing any symptoms. As shown in the work by Adikini et al. (2011), the symptoms occur days after the colonization of the plant tissue by the pathogen, depending on the variety and bacteria isolate.

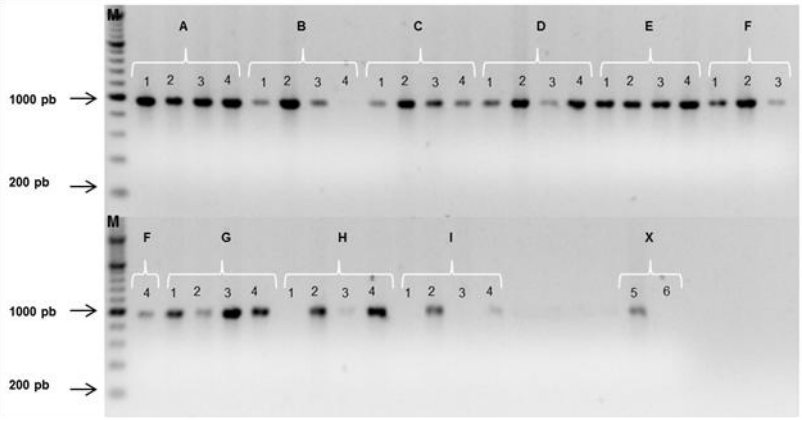

Figure 4. 1% agarose gel. PCR product from the detection test in different fragments of the stem. A: Hybrid 9624-09; B: BRS Caipira; C: Cigana Preta; D: BRS Tapioqueira; E: BRS Kiriris; F: BRS Poti Branca; G: Hybrid 98150-06; H: Solangor; I: BRS Verdinha; X: Control. 1= 1 cm in the base of the stem; 2 = 1 cm at the point of inoculation; 3 = 8 cm above the inoculation point; 4 = 16 cm above the inoculation point; 5 = Positive control (*Xpm*, isolate IBSBF 278); 6 = Negative control (Mix without DNA). M: 200 bp DNA ladder.

These detection results from different points of the stem and in symptomatic and asymptomatic tissue allow for an efficient detection of *Xpm* since the main form of planting cassava is by stem cuttings, which could be infected with *Xpm*, contributing to disease dissemination across different fields or even with long distance transportation. The use of different stem points for the indexation process proves to be mostly applicable to guarantee plant health before the transport of cuttings to areas free of the disease in the cassava planting process.

Specificity of the primers in multiplex PCR

The specificity of the primer pair using the different bacterial species and pathovars listed in Table 1 was tested by conventional multiplex PCR using primers XV / XK and 16S8F / 16S1492R (Figure 5A), XV / XK_MOD and 16S8F / 16S1492R (Figure 5B), and a single PCR with the universal 16S primers 16S8F / 16S1492R (control) (Figure 5C). Multiplex PCR approaches with internal controls have effectively been used in the diagnosis of various *Xanthomonas* species (Berg, Tesoriero, & Hailstones, 2005; Robène-Soustrade et al., 2010; Adriko et al., 2012).

The results show that the fragment corresponding to the 16S rRNA gene produced bands of approximately 1.2 kb in all samples both in multiplex reactions (Figure 5A and B) and in the reaction with only this primer (Figure 5C). For the reactions using the primer pair XV / XK developed by Verdier et al. (1988), there was no amplification of the samples used. Contrary to what was verified by Wonni et al. (2015), the expected DNA fragment was obtained from all the Xpm strains. However, five nonpathogenic Xpm strains were tested with the primer pair from the assay and produced no amplification product (Verdier et al., 1998).

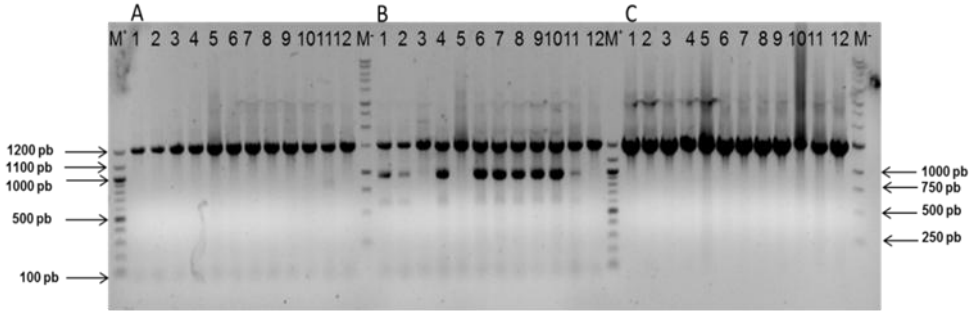

Figure 5. PCR products from the sensitivity test with bacterial isolates from different genera and species. (A): Isolates of *X. phaeoli* pv. *manihotis*. 1: IBSBF 1994; 2: IBSBF 291; 3: 328; 4: IBSBF 436*;* 5: 726*;* 6-10: CBEMF-9 isolate (with DNA extraction conducted at different times)*;* 11: IBSBF 278 and 12: *Xanthomonas axonopodis* pv. *passiflorae* (CBEMF-8), with primers 16S rRNA e XV / XK. (B): 1: IBSBF 1994; 2: IBSBF 291; 3: 328; 4: IBSBF 436*;* 5: 726*;* 6-10: CBEMF-9 isolate (with DNA extraction conducted at different times)*;* 11: IBSBF 278 and 12: *Xanthomonas axonopodis* pv. *passiflorae* (CBEMF 8), with primers 16S rRNA and XV / XK_MOD. (C): 1: IBSBF 1994; 2: IBSBF 291; 3: 328; 4: IBSBF 436*;* 5: 726*;* 6-10: CBEMF-9 isolate (with DNA extraction conducted at different times)*;* 11: IBSBF 278 and 12: *Xanthomonas axonopodis* pv. *passiflorae* (CBEMF 8), with primer 16S rRNA. M*: 100 bp DNA Ladder; M: 1 kb DNA ladder.

For the primer pair XV / XK_MOD, a fragment of approximately 1 kb was amplified for *Xpm* isolates but not for the other bacteria samples (Figure 4B). However, in some samples, primer pair XV / XK_MOD showed a high degree of specificity with the ability to detect the pathogen with high reliability when compared with XV / XK, as noted above.

According to the results, the use of specific primers for the 16S rRNA gene is a control for confirmation of bacterial DNA, which confirms that the lack of amplification for the combination XV / XK was not due to the quality of DNA or problems with the PCR mix. For *Xanthomonas campestris pv. musacearum* (*Xcm*), Adriko et al. (2012), indicates that efficient multiplexing with ribosomal primers allows the immediate quality assessment of negative results regarding the *Xcm* amplicon.

Therefore, the multiplex PCR assay described here is a reliable and sensitive procedure for detecting and identifying *Xpm* in cassava for the direct quality assessment of results.

Efficient diagnosis measures can be crucial to prevent the spread of disease (Miller et al., 2009). Given the great economic and social importance that cassava has worldwide and the losses caused by the occurrence of bacterial blight caused by *Xpm*, rapid detection of this pathogen can significantly reduce the damage caused. Thus, the use of specific primers can help in the application of phytosanitary measures, enabling rapid and reliable diagnosis of the pathogen to accelerate the implementation of controls and avoid the establishment of the disease in disease-free areas.

Conclusion

The combination of the forward primer (XV) and the modified primer (XK_MOD) was effective in amplifying the specific fragment for *Xpm* from DNA from in vitro cultures and total DNA from infected plant tissue, both symptomatic and asymptomatic.

It is possible to estimate the systemic movement of bacteria using the combination XV / XK_MOD since amplification was detected at different points of the stem.

The diagnosis of *Xpm* using PCR was efficient and can be used by Phytopathological clinics and plant defence agencies to prevent disease dissemination and ensure the purity of planting material.

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