

Detecting *Xanthomonas axonopodis* pv. *manihotis* in cassava true seeds by nested polymerase chain reaction assay

S. Ojeda and V. Verdier

Abstract: Cassava bacterial blight, caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is a particularly destructive disease in South America and Africa. Because of quarantine procedures, international exchange of cassava germplasm is limited and occurs through true seeds or material propagated in vitro. The success of a cassava-seed certification program depends on the availability of reliable tests to detect the pathogen in true seeds and vegetative planting materials. We developed a nested polymerase chain reaction assay to detect the pathogen in cassava true seeds. The internal primers directed the amplification of a fragment of 509 base pairs in all 12 *Xam* strains tested, whereas no amplification product was obtained from any of the 17 strains of other *Xanthomonas* pathovars tested. Nested polymerase chain reaction worked well for *Xam* detection from cultured cells from artificially inoculated seeds and naturally infected seeds. The best detection level allowed to detect a presence as low as one to two viable cells per reaction. This technique was specific, sensitive, and rapid for detecting *Xam* in cassava true seeds.

Key words: *Xanthomonas axonopodis* pv. *manihotis*, cassava bacterial blight, nested polymerase chain reaction, detection, true seeds, bacteria.

Résumé : La bactériose vasculaire du manioc, causée par le *Xanthomonas axonopodis* pv. *manihotis*, est une maladie particulièrement destructive en Amérique du Sud et en Afrique. L'échange de matériel végétal au niveau international est limité par des mesures de quarantaine et ne peut se faire qu'à l'aide de plantules issues de graines ou de matériel multiplié in vitro. La réussite du programme de certification des semences de manioc dépend de la disponibilité de tests fiables permettant de détecter l'agent pathogène dans les graines et dans le matériel végétal destiné à la plantation. Nous avons développé une réaction en chaîne de la polymérase nichée pour détecter l'agent pathogène dans les graines. Les amorces internes mises au point amplifient un fragment de 509 paires de bases dans les 12 souches de *Xam* testées, tandis qu'aucune amplification n'est obtenue à partir des 17 souches d'autres pathovars de *Xanthomonas*. La réaction en chaîne de la polymérase nichée permet la détection de *Xam* à partir de cultures bactériennes et de graines infectées artificiellement et naturellement. Le meilleur niveau de détection permettait de détecter une présence aussi faible que une à deux cellules viables par réaction. Les résultats obtenus montrent que cette technique est spécifique, sensible et rapide pour détecter *Xam* dans les graines de manioc.

Mots clés : *Xanthomonas axonopodis* pv. *manihotis*, bactériose vasculaire du manioc, réaction en chaîne de la polymérase nichée, détection, semences, bactéries.

Introduction

Cassava, *Manihot esculenta* Crantz, is one of the major sources of food in tropics (Lozano and Sequeira 1974). World production of cassava is estimated to be 120×10^6 t annually (Lozano 1986). About 80% of the cassava is consumed by human beings and constitutes the principal carbohydrate source for more than 500×10^6 people in developing countries (Cock 1985). Cassava bacterial blight, caused by *Xanthomonas axonopodis* Arthaud-Berthet Starr pv. *manihotis* (*Xam*), is a particularly destructive disease in South America and Africa. This vascular disease has a wide

spectrum of symptoms, including angular leaf spots, blight, production of exudates, wilting, and dieback (Maraite 1993). Seed yield is also reduced by this disease (Boher and Verdier 1994; Lozano 1986). Cassava is propagated mainly by planting pieces of stem, and the pathogen can be disseminated through infected planting material. *Xam* is also seedborne and is carried either on the seed coat or in the embryo (Daniel and Boher 1981a; Lozano and Jayasinghe 1983). Seed contamination is an important means of survival for this bacterium (Persley 1979; Lozano et al. 1986).

The disease can be controlled through cultural practices, including the use of uninfected planting materials and resistant cultivars (Lozano 1986). Although the production of healthy planting materials can reduce the incidence of the disease, this method is limited because *Xam* can survive in tissue without causing symptoms (Boher and Verdier 1994). Because of quarantine procedures, international exchange of cassava germplasm is limited and occurs through true seeds or material propagated in vitro (Lozano 1986). The success

Accepted February 21, 2000.

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of a cassava seed certification program, however, depends on the availability of reliable tests to detect the pathogen in true seeds.

Current methods to identify and detect *Xam* rely on isolating the bacterium and conducting immunoassays of tissue extracts, such as ELISA (enzyme-linked immunosorbent assays), but are not entirely specific because of cross-reactions (Daniel and Boher 1981b; Elango and Lozano 1980). Monoclonal antibodies against extracellular polysaccharides (LPS) of *Xam* have been also produced but they are not highly specific to the pathogen (Boher et al. 1997). A PCR (polymerase chain reaction) assay was developed for detecting cassava bacterial blight in cassava leaves and stems (Verdier et al. 1998) but not true seeds. Recently, a dot-blot assay was developed to detect *Xam* in cassava fruits and seeds (Verdier and Mosquera 1999).

Molecular techniques such as PCR and IC PCR (immunocapture PCR) have been reported as highly efficient for identifying and detecting plant pathogens such as *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *citri*, and *Xylella fastidiosa* (Hartung et al. 1993; Hartung et al. 1996; Pooler et al. 1997; Toth et al. 1998). Nested PCR is a PCR-based assay for which a second round of amplification is conducted by using primers internal to the first amplification product (Pooler et al. 1997). The advantage of using this technique is improved detection compared with the PCR technique. It also retains the specificity of previous assays (Pooler et al. 1997).

Previous assays, using the PCR primer pair XV–XK, amplified a 898-bp (base pair) fragment in all *Xam* strains studied for both DNA samples and planting materials (leaves and stems naturally and artificially inoculated) (Verdier et al. 1998). For the present study, we developed a pair of internal primers and established the conditions of a nested PCR for *Xam* detection. Our objective was to develop a rapid and sensitive method for detecting *Xam* in cassava true seeds.

Materials and Methods

Bacterial strains

All the strains used are listed in Table 1. To test primer specificity, 12 *Xam* strains and 17 strains representing other pathovars of *Xanthomonas* were used. Strain OrstX27 was routinely cultured at 30°C on yeast–peptone–glucose agar (YPG) (5 g glucose, 5 g yeast extract, 5 g peptone, and 15 g agar per liter) for performing detection assays to evaluate the sensitivity level. Freshly grown bacteria on YPG agar from a 24-h culture was used for PCR and nested-PCR assays.

PCR conditions

Primers for the nested-PCR assay were designed with the software Primer Premier version 4.0, based on the sequence of the amplification product from the first round of PCR. The primers were commercially obtained from Operon Technologies, Inc. (Alameda, Calif.). Nucleotide sequences of the internal primers used in this study are 5'-AAC-AGT-AAG-TCG-GTG-TCG-CC-3' (primer XV2) and 5'-TCG-TTG-CCG-TGG-CTA-CC-3' (primer XK2). PCR assays were performed as described by Verdier et al. (1998).

Nested-PCR assays were performed with a DNA thermal cycler (PTC-100, MJ Research, Watertown, Mass.). Nested-PCR reactions contained 1.5 mM MgCl₂, primer (12.5 pmol), 200 μM nucleotides, and 1.25 units of *Taq* DNA polymerase per 50 μL of reaction. The nested-PCR temperature profile comprised an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min, and a final extension at 72°C for 5 min.

Two DNA concentrations (20 and 200 ng/μL) were tested in the first round of PCR to evaluate the best for use in the second round of PCR. From the PCR product, a 10-fold dilution was used to perform the nested-PCR assay with the strains shown in Table 1.

To determine the detection sensitivity level with cultured cells, bacteria from a 24-h culture on YPG agar plates were resuspended in sterile water and centrifuged for 5 min at 12 000 rpm. The supernatant was discarded and the pellet resuspended in 300 μL of distilled sterile water. The final suspension was serially diluted in 10-fold series in distilled sterile water. Dilutions were plated on YPG agar and the colonies that developed were counted. Before PCR, bacterial suspensions were boiled for 10 min, then 10 μL of each dilution was used as a source of DNA template for the PCR reaction. For nested PCR, 10 μL of the PCR amplification product were placed in a fresh sterile tube with the nested-PCR reaction mix for the second round of amplification. Negative control reactions (either no DNA or sterile water) were run in all experiments. The PCR as well as the nested-PCR amplification products were analyzed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and photographed using the Stratagene Eagle Eye II Still Video System.

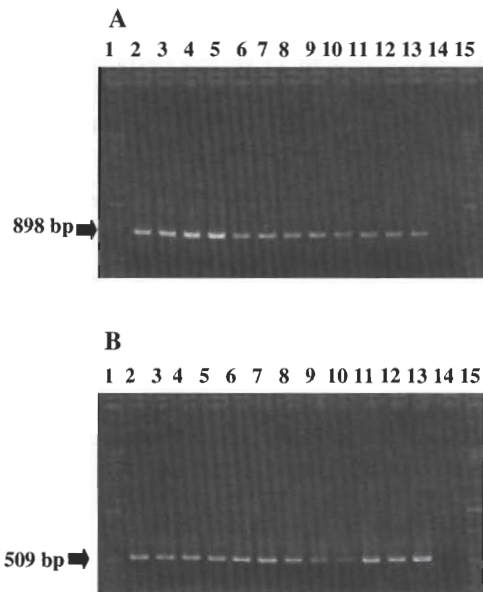
Detecting *X. axonopodis* pv. *manihotis* in artificially inoculated true seeds

Healthy seeds from the cassava M COL 1522 cv. were inoculated as follows: an inoculum of 10⁸ cfu/mL (optical density at 600 nm = 0.1) was prepared from a fresh culture as described above. Three lots of 10 seeds each were scarified and then washed in sterile water. Each lot of seeds was inoculated by placing it in the bacterial suspension and by applying vacuum for 30 min. The inoculated seeds were washed twice by shaking for 30 min in distilled sterile water (1.5 mL/seed), and then individually macerated in distilled sterile water (1.5 mL/seed). All macerated seeds from each lot (10 seeds) were collected in one tube. The first seed washes were discarded. The second seed washes, as well as the seed macerates, were serially diluted in distilled sterile water and plated on YPG. Plates were incubated at 30°C for 48 h and the colonies that developed were counted. From the seed washes and macerates, 10 μL of each dilution were used as a source of DNA template and incubated at 95°C for 10 min. Proteinase K, 1 μL (10 mg/mL), was added and samples were incubated at 55°C for 12 min. Finally, the samples were kept at 20°C for 3 min before adding *Taq* polymerase. The PCR profile was as described above. For nested PCR, 10 μL of each PCR product were used as a source of DNA template. Purified DNA from strain OrstX27 was used as positive control; sterile water and healthy washed or macerated seeds were

Table 1. PCR and nested-PCR assays on strains of *Xanthomonas* bacteria.

| Strain | Origin | PCR | Nested PCR |
|--------------------------------------------------------------------|---------------|-----|------------|
| <i>X. axonopodis</i> pv. <i>manihotis</i> | | | |
| CIO 324, CIO 325 CIO 347, CIO 358 | Brazil | + | + |
| CIO 46, CIO 503, CIO 510, CIO 513, CIO 514, CIO 521, CIO 523 | Colombia | + | + |
| OrstX27 | Togo | + | + |
| <i>X. axonopodis</i> pv. <i>aracearum</i> , NCPPB 2832 | Guadeloupe | - | - |
| <i>X. axonopodis</i> pv. <i>campestris</i> , NCPPB 528, CFBP 10412 | UK | - | - |
| <i>X. axonopodis</i> pv. <i>citri</i> , CFBP 1814 | Réunion | - | - |
| <i>X. campestris</i> pv. <i>euphorbiae</i> , LMG 863 | Sudan | - | - |
| <i>X. axonopodis</i> pv. <i>fuscans</i> , CIAT 266 | Argentina | - | - |
| <i>X. axonopodis</i> pv. <i>glycines</i> , Orst1144 | Congo | - | - |
| <i>X. axonopodis</i> pv. <i>incanae</i> , CFBP 1438 | USA | - | - |
| <i>X. axonopodis</i> pv. <i>malvacearum</i> , Orst57 | Côte d'Ivoire | - | - |
| <i>X. axonopodis</i> pv. <i>mangifereindicae</i> , CFBP 1716 | Réunion | - | - |
| <i>X. axonopodis</i> pv. <i>pelargonii</i> , NCPPB 2985 | New Zealand | - | - |
| <i>X. axonopodis</i> pv. <i>phaseoli</i> , XCP 095 | Colombia | - | - |
| <i>X. axonopodis</i> pv. <i>poinsetticola</i> , UPB 073 | India | - | - |
| <i>X. axonopodis</i> pv. <i>ricini</i> , UPB 075 | Ethiopia | - | - |
| <i>X. axonopodis</i> pv. <i>vignicola</i> , UPB 040 | Niger | - | - |
| <i>X. oryzae</i> pv. <i>oryzae</i> , CFBP 1948 | Cameroon | - | - |
| <i>X. oryzae</i> pv. <i>oryzicola</i> , CFBP 2286 | Not known | - | - |

Fig. 1. PCR (A) and nested-PCR (B) amplification products of DNA extracted from 12 strains of *X. axonopodis* pv. *manihotis*. Lane 1, 1-kb (kilobase) ladder (GIBCO BRL); lane 2, OrstX27; lanes 3–13: 3, CIO 46; 4, CIO 359; 5, CIO 332; 6, CIO 339; 7, CIO 324; 8, CIO 325; 9, CIO 358; 10, CIO 514; 11, CIO 510; 12, CIO 503; 13, CIO 513; lane 14, negative control (distilled water); lane 15, 1-kb ladder.

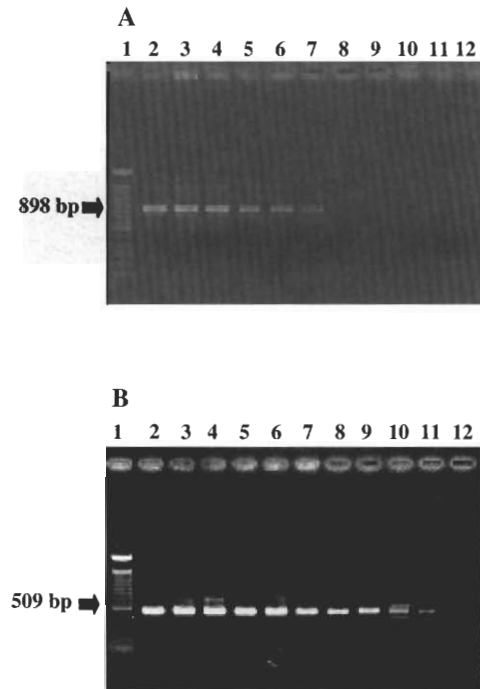


used as negative controls. All the PCR experiments were repeated at least twice.

Detecting *X. axonopodis* pv. *manihotis* in embryos from artificially inoculated seeds

Ten lots of 10 seeds each were inoculated, then washed as described above. The seeds were opened with a pair of

Fig. 2. PCR (A) and nested-PCR (B) amplification products from cultured cells of *X. axonopodis* pv. *manihotis* (*Xam*) strain OrstX27. Lane 1, 100-bp ladder (GIBCO BRL); lane 2, positive control of DNA from (*Xam*) strain OrstX27; lanes 3–11 (cfu/10 μ L): 3, 1.4×10^9 ; 4, 1.4×10^8 ; 5, 1.4×10^7 ; 6, 1.4×10^6 ; 7, 1.4×10^5 ; 8, 1.4×10^4 ; 9, 1.4×10^3 ; 10, 1.4×10^2 ; 11, 1.4×10^1 ; lane 12, negative control (distilled water).

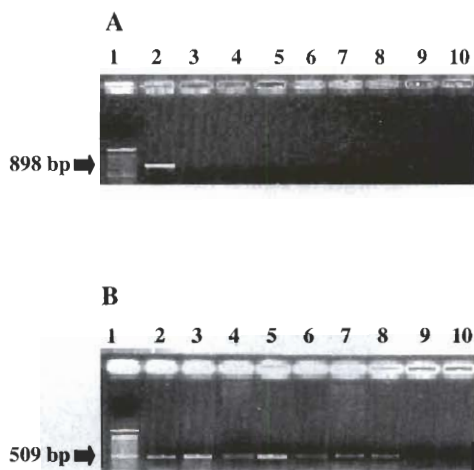


pincers to take out the embryos, holding the seed from the part where the embryo is placed. Ten embryos from each lot were placed in a sterile Eppendorf tube, then macerated with 100 μ L of distilled sterile water. The PCR and nested

Table 2. Detection level of *Xam* with PCR and nested-PCR assays.

| Bacterium dilution | <i>Xam</i> recovered on YPG agar (cfu/mL) | PCR | | Nested PCR | |
|--------------------|-------------------------------------------|--------|--------------------------|------------|--------------------------|
| | | Result | Detection level (cfu/mL) | Result | Detection level (cfu/mL) |
| 0 | 1.4×10^{11} | + | 1.4×10^9 | + | 1.4×10^9 |
| -1 | | + | 1.4×10^8 | + | 1.4×10^8 |
| -2 | | + | 1.4×10^7 | + | 1.4×10^7 |
| -3 | | + | 1.4×10^6 | + | 1.4×10^6 |
| -4 | | + | 1.4×10^5 | + | 1.4×10^5 |
| -5 | | - | - | + | 1.4×10^4 |
| -6 | | - | - | + | 1.4×10^3 |
| -7 | | - | - | + | 1.4×10^2 |

Fig. 3. PCR (A) and nested-PCR (B) amplification products from artificially inoculated seeds with *X. axonopodis* pv. *manihotis* (*Xam*) strain OrstX27. Lane 1, 100-bp ladder (GIBCO BRL); lane 2, positive control of DNA from (*Xam*) strain OrstX27; lanes 3–5, seed wash SW1–SW3, containing respectively 9.6×10^2 , 8.8×10^1 , and 9.3×10^1 cfu per reaction, respectively; lanes 6–8, seed macerate SM1–SM3, containing respectively 1.7, 1.2, and 5.6 cfu per reaction; lane 9, negative control (distilled water); lane 10, noninoculated washed seeds.



PCR were conducted as described above; 10 μ L of each sample were used as DNA template to perform both assays. Controls were DNA from strain OrstX27, distilled sterile water, and embryos from uninoculated seeds. The PCR experiments were repeated at least twice for each sample.

Detecting *X. axonopodis* pv. *manihotis* in seed samples collected from infected fields

Cassava fruits collected from infected fields at Villavicencio (Colombia) were dried at room temperature for 2 weeks. Seeds were then taken out of each fruit. Ten lots of 10 seeds each were washed in distilled sterile water (1.5 mL/seed), serially diluted, then plated on YPG agar plates. The colonies that developed were counted. From each sample, 10 μ L were used as DNA template for PCR after being treated as indicated above. Samples of 10 μ L of each PCR product were used for nested PCR. All the PCR experiments were repeated at least twice for each sample.

Results

Primer specificity

The primers XV and XK for PCR assays directed the amplification of a 898-bp nucleotide product (Fig. 1A). The primers XVS2 and XKS2 directed the amplification of a 509-bp product in DNA from 12 different strains of *Xam* (Table 1; Fig. 1B). No amplification product was obtained when the DNA of 17 strains of other pathovars of *Xanthomonas* was tested for internal primer specificity (Table 1).

Sensitivity of the nested-PCR assay of cultured cells

Using either 20 or 200 ng/ μ L of DNA, the expected amplification product was obtained. Samples of 20 ng/ μ L of DNA were then used for all assays. Dilution series of freshly cultured cells of *Xam* strain OrstX27 yielded a detection limit of 14 viable cells per reaction as determined by dilution plating on YPG agar (Fig. 2). Comparing the detection level obtained with PCR and nested PCR, we found that the nested PCR was more sensitive than the PCR for detecting *Xam* from freshly cultured cells (Table 2).

Detection of *X. axonopodis* pv. *manihotis* in artificially inoculated true seeds

No amplification product was obtained from seed materials for the first round of PCR, whereas, for nested PCR, the three different seed lots, including washes (SW1, SW2, SW3) and macerates (SM1, SM2, SM3), were positive (Fig. 3). These results show 100% positive results for nested PCR. The minimum number of viable cells detected by nested PCR was 88 per reaction with seed washes and one to two viable cells per reaction with macerated seeds (Fig. 3; Table 3). No amplification product was obtained from healthy uninoculated seeds, analyzed as controls (data not shown).

Detection of *X. axonopodis* pv. *manihotis* in embryos from artificially inoculated seeds

No amplification products were observed from embryo tissues with the PCR assay, while 5 of the 10 lots were positive in the nested-PCR assay (data not shown). *Xam* colonies were not observed on YPG agar. No amplification product was obtained for healthy embryos from uninoculated seeds used as control.

Table 3. Detection levels of *Xanthomonas axonopodis* pv. *manihotis* in PCR assays and nested-PCR assays of inoculated cassava (*Manihot esculenta* Crantz) seeds.

| Seed lot type | No. | <i>Xam</i> recovered on YPG agar (cfu/mL) | PCR result | Nested PCR | |
|----------------|-----|-------------------------------------------|------------|------------|--------------------------|
| | | | | Result | Detection level (cfu/mL) |
| Seed washes | 1 | 9.6×10 ⁴ | – | + | 9.6×10 ² |
| | 2 | 8.8×10 ⁴ | – | + | 8.8×10 ¹ |
| | 3 | 9.3×10 ⁴ | – | + | 9.3×10 ¹ |
| Seed macerates | 1 | 1.7×10 ⁴ | – | + | 1.7 |
| | 2 | 1.2×10 ⁴ | – | + | 1.2 |
| | 3 | 5.6×10 ⁴ | – | + | 5.6 |

Table 4. Detection levels of *Xanthomonas axonopodis* pv. *manihotis* in PCR and nested-PCR assays in naturally infected cassava (*Manihot esculenta* Crantz) seeds.

| Sample lots | Bacterium concentration (cfu/mL) | PCR result | Nested PCR | |
|-------------|----------------------------------|------------|------------|--------------------------|
| | | | Result | Detection level (cfu/mL) |
| 1 | 6.2×10 ⁴ | – | + | 6.2×10 ² |
| 2 | 1.2×10 ⁴ | + | + | 1.2×10 ² |
| 3 | 4.2×10 ⁴ | – | + | 4.2×10 ² |
| 4 | 3.4×10 ⁴ | – | + | 3.4×10 ² |
| 5 | 1.2×10 ⁴ | + | + | 1.2×10 ² |
| 6 | 7.0×10 ⁴ | – | + | 7.0×10 ² |
| 7 | 2.6×10 ⁴ | + | + | 2.6×10 ² |
| 8 | 4.8×10 ⁴ | – | + | 4.8×10 ² |
| 9 | 8.6×10 ⁴ | – | + | 8.6×10 ² |
| 10 | 3.8×10 ⁴ | – | + | 3.8×10 ² |

Detection of *X. axonopodis* pv. *manihotis* in true seeds collected from infected fields

Washes from 10 seed lots were tested by PCR and nested PCR. Results indicated that 5.7% of the samples were positive for PCR and that 35.7% were positive for nested PCR. Samples that were positive for PCR were also positive for nested PCR. The number of viable cells detected for nested PCR ranged from 1.2×10^2 to 8.6×10^2 cells per reaction. (Table 4; Fig. 4). The attempts to recover *Xam* from the seed washes from naturally infected seeds in YPG agar were successful. However, the presence of background microflora made determining the exact number of viable cells difficult.

Discussion

New PCR primers XVS2 and XKS2 were developed in this study for the specific and sensitive detection of *Xam* in cassava true seeds. We developed a nested-PCR assay, using the sequence from a unique *Xam* PCR product (Verdier et al. 1998). The internal primers directed the amplification of a 509-bp nucleotide product that was obtained from all *Xam* strains tested. No amplification product was obtained with any other bacterial strains evaluated.

Using cultured cells, nested-PCR assay improved our single-stage PCR by about four times cultured cells per reaction. This experiment was repeated several times with the same *Xam* strain, and results were highly reproducible. Nevertheless, the detection level achieved by the PCR assay with cultured cells is lower than the one previously obtained

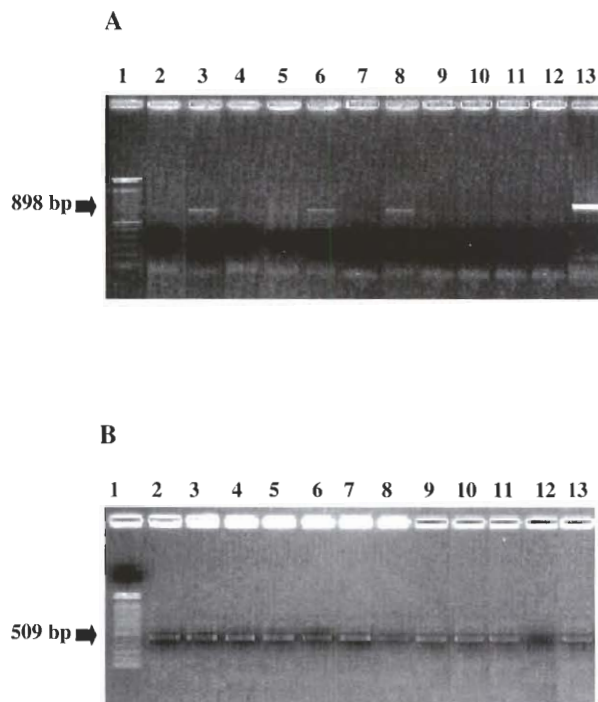
with strain CIO 46 (Verdier et al. 1998). More strains must be tested to clarify whether differences in the level of detection obtained with the first round of PCR result from the type of strain used.

The finding that *Xam* is consistently associated with true (i.e., sexual) seeds, which have been stored for several months, suggests a potential for transmitting the pathogen by seed (Persley 1979). The pathogen is usually located in the embryo in a dormant-like stage that is activated after germination (Lozano and Jayasinghe 1983). In this study, we confirmed the presence of *Xam* in true seeds. No DNA product was amplified from the first round of PCR, whereas in the second round of PCR (nested PCR), the pathogen was successfully detected in either seed washes, macerates, or embryos from artificially inoculated seeds. The use of a nested PCR thus increases sensitivity of detection.

Recently, a dot-blot assay, using a DNA fragment unique to *Xam* (Verdier and Mosquera 1999), successfully detected the pathogen in cassava fruits, true seeds, and embryos that were naturally infected. Usually, when compared with PCR assays, the dot-blot assay was 10 to 100 times less sensitive (Verdier and Mosquera 1999). Nevertheless, in our study, all the samples of either washes or macerates of inoculated seeds were refractory to PCR amplification while the pathogen was only detected by nested PCR.

The absence of DNA amplification by, or the low sensitivity of, PCR with plant materials may be caused by the presence of PCR inhibitors such as phenolic compounds (Audy et al. 1996). These compounds are present in cassava tissues and they might interfere with DNA polymerase ac-

Fig. 4. PCR (A) and nested-PCR (B) amplification products from cassava (*Manihot esculenta* Crantz) seeds collected from naturally infected fields. Lane 1, 100-bp ladder (GIBCO BRL); lanes 2–11, seed wash from samples 1–10, containing respectively (cfu): lane 2, 6.2×10^2 ; 3, 1.2×10^2 ; 4, 4.2×10^2 ; 5, 3.4×10^2 ; 6, 1.2×10^2 ; 7, 7×10^2 ; 8, 2.6×10^2 ; 9, 4.8×10^2 ; 10, 8.6×10^2 ; 11, 3.8×10^2 ; lane 12, negative control (distilled sterile water); lane 13, positive control of DNA from *X. axonopodis* pv. *manihotis* strain OrstX27.



tivity. Other chemicals such as copper hydroxide may also act as PCR inhibitors (Hartung et al. 1996). The effects of these inhibitors can be prevented by using products such as proteinase K, polyvinylpyrrolidone, or sodium ascorbate (Leite et al. 1995). For the present study, we added proteinase K to the samples to control PCR inhibition. The use of techniques like immunocapture before the PCR assay can also help eliminate the presence of inhibitors (Hartung et al. 1996; Pooler et al. 1997).

Positive results were obtained with the nested-PCR assay when evaluating seeds from infected fields. Also viable bacteria could be detected by the plating assay. We observed the presence of considerable background microflora, which constitutes a major limitation in the detection of plant-pathogenic bacteria by the plating method. However, the specificity of the DNA amplification procedure can overcome this problem (Leite et al. 1995). *Xam* cells probably do not survive competition with saprophytes very well. This hypothesis is supported by the observation that only few viable cells grew from most of the seed samples collected from infected fields, while many of the samples were positive in the nested-PCR assay. To eliminate background microflora, a semi-selective medium for *Xam* can be used (Fessehaie et al. 1999). Noninfected seeds were also tested by the nested-PCR assay and no amplification products were obtained. Cells do not need to be viable for successful PCR amplification, but intact target nucleic acid sequences

are required. In the environment, nucleic acids of nonviable cells are probably degraded quickly, as demonstrated for boiled *Escherichia coli* cells added to natural pond water (Josephson et al. 1993).

Seeds are used for international exchange of cassava germplasm and, frequently, few seeds are available for any given cultivar. The nested-PCR procedure is simple and fast and can be easily implemented to certify seed lots. Furthermore, the seed-washing procedure developed is nondestructive, and the bacterium DNA does not have to be extracted before processing the seeds, allowing the material to be tested in only one day. With its specificity and sensitivity, the nested-PCR procedure described is a potentially reliable procedure for detecting and identifying the cassava bacterial blight pathogen in cassava true seeds.

Acknowledgements

We would like to thank several people at CIAT for supporting our study, specifically W. Roca, G. Mosquera for her assistance at the beginning of this study, N. Morante for providing us with cassava seeds, S. Restrepo for her helpful discussions, and V. Jorge and W. Gaitán for their help during sampling. We also thank Elizabeth de Páez for editing. Financial support was provided to S. Ojeda by the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología Francisco José de Caldas (COLCIENCIAS) in the form of a junior scientist grant.

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