Multiplex Nested PCR for Detection of *Xanthomonas axonopodis* pv. allii from Onion Seeds

Isabelle Robène-Soustrade,* Delphine Legrand, Lionel Gagnevin, Frédéric Chiroleu, Annie Laurent, and Olivier Pruvost

CIRAD, UMR Peuplements Végétaux et Bioagresseurs en Milieu Tropical CIRAD—Université de la Réunion, Pôle de Protection des Plantes, 7 Chemin de l’Irat, 97410 Saint Pierre, La Réunion, France

Received 6 November 2009/Accepted 22 February 2010

Bacterial blight of onion (BBO) is an emerging disease that is present in many onion-producing areas. The causal agent, *Xanthomonas axonopodis* pv. allii, is seed transmitted. A reliable and sensitive diagnostic tool for testing seed health is needed. Detection of *X. axonopodis* pv. allii was achieved using a multiplex nested PCR assay developed using two randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) sequences corresponding to pilus assembly genes (*pilV* and *pilX*) and the *avrRxv* gene, respectively. The multiplex nested PCR was used with a large collection of *X. axonopodis* pv. allii strains pathogenic to onion and/or other *Allium* species isolated in different regions of the world. The internal primers used in the multiplex PCR assay directed amplification for all 86 *X. axonopodis* pv. allii strains tested, resulting in a 401-bp amplicon, a 444- to 447-bp amplicon, or both amplicons, depending on the strain. No amplification was obtained for 41 unrelated phytopathogenic bacteria and for 14 saprophytic bacteria commonly isolated from onion leaves and seeds. Most *Xanthomonas* strains also did not produce amplicons, except for nine strains classified in *X. axonopodis* genetic subgroup 9.1 or 9.2 and not pathogenic to onion. Nevertheless, sequence signatures distinguished most of these strains from *X. axonopodis* pv. allii. The assay detected *X. axonopodis* pv. allii in seed lots with contamination levels of $5 \times 10^5$ CFU g$^{-1}$ or higher. The sensitivity threshold of the multiplex nested PCR assay was found to be 1 infected seed in 27,340 seeds. This PCR-based assay should be useful for certifying that commercial seed lots are free of this important seed-borne pathogen.

Onion (*Allium cepa* L.) is grown worldwide, covering a total area of roughly 6.7 million acres in at least 125 different countries. The total annual bulb production in 2007 was estimated to be 64.5 million tons (http://apps.fao.org/faostat/). Bulb crops. The total annual bulb production in 2007 was estimated at the point of initial infection. Leaf tip death and blight reduce the photosynthetic capacity of plants, leading to a reduction in bulb size. Yield losses ranging from 20 to 50% have been recorded under conditions conducive to efficient development of disease (28, 50).

Pathogenicity tests have indicated that the host range of *X. axonopodis* pv. allii includes several *Allium* species (garlic [*A. sativum* L.], Welsh onion [*A. fistulosum* L.], shallot [*A. cepa* var. *ascalonicum* BACKER], chive [*A. schoenoprasum* L.], and leek [*A. porrum* L.]) (41), as well as at least two *Cirrus* species (11). Worldwide, most outbreaks have been reported on onion, but outbreaks have also affected leek and garlic in the Mascarene Archipelago (33) and Welsh onion in Japan (17). Compared to some *Xanthomonas* pathogens, *X. axonopodis* pv. allii is phenotypically and genetically diverse (11, 12, 33, 41).

Onion seeds originating from diseased fields were identified as a possible pathway for *X. axonopodis* pv. allii transmission (42). Inoculum associated with seeds contaminated at a rate of 4/10,000 has been used as the primary inoculum in field epidemiological studies (43). Recently, onion seeds were identified as the most probable pathway for the introduction of the pathogen into Réunion Island from the neighboring country Mauritius (33). Currently, the pathogen is isolated from onion seeds or from plant material using semiselective media (42, 51) and a subsequent identification step consisting of pathogenicity tests and/or molecular typing techniques (12, 33). This approach is time-consuming, and false-negative results can occur when population densities of the pathogen are low because of bacterial microbiota associated with seeds.

For several pathosystems involving xanthomonads, it has been shown that long-distance spread of pathogens can occur through contaminated seeds (53), and sensitive methods to certify that seed lots are pathogen free have been developed (20). Therefore, making highly specific and sensitive PCR-based diagnostic tools available for *X. axonopodis* pv. allii is a priority. PCR-based techniques have been reported to be...
highly efficient for detecting and identifying xanthomonads from seeds, such as *X. campestris* pv. carotae (23), *X. oryzae* pv. oryzae (46), *Xanthomonas* pathogens that cause cereal leaf streak (21), and *X. axonopodis* pv. manihotis (31). In the case of *X. axonopodis* pv. manihotis, a nested PCR (N-PCR) protocol was used to enhance the sensitivity and specificity of detection. In addition, multiplex PCR protocols have been developed in order to detect several pathogens (13) or genetically heterogeneous strains of a single pathovar (24, 56) simultaneously.

In this study we developed PCR primers specific to *X. axonopodis* pv. *allii* for sensitive and specific detection of all strains of this pathovar, and we evaluated the reliability of the method for detection of the pathogen in contaminated onion seed lots.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and DNA extraction.** The 86 *X. axonopodis* pv. *allii* strains used in this study and related information are shown in Table S1 in the supplemental material. Bacterial strains belonging to different *Xanthomonas* pathovars (60 strains) and species (25 strains) and to different genera (16 strains) and saprophytic bacteria isolated from onion (18 strains) were used for specificity analyses (see Table S2 in the supplemental material). The saprophytic bacteria were identified at the genus level using the Biolog system (6, 42). Strains were stored at −80°C on beads in cryovials (Microban; Prolab Diagnostics, Austin, TX) and/or as lyophilisates for long-term storage. Strains were routinely grown on YPGA (yeast extract, 7 g liter−1; peptone, 7 g liter−1; glucose, 7 g liter−1; agar, 18 g liter−1; pH 7.2) at 28°C. Strains that grew poorly on YPGA were cultivated on modified Wilbrink medium (40). One-day-old bacterial cultures were used for PCR assays and pathogenicity tests. DNA was extracted from 2 ml bacterial cultures (16 h of incubation with agitation at 28°C in YP broth [yeast extract, 7 g liter−1; peptone, 7 g liter−1; pH 7.2]) using a DNeasy blood and tissue kit (Qiagen, Courtaboeuf, France) and following the manufacturer’s instructions. DNA concentrations were estimated by fluorometry (TKO 100 fluorometer; Hoefer, San Francisco, CA).

**Selection of randomly amplified polymorphic DNA (RAPD) markers.** PCR assays were performed using a PEr6000 cycler (Applied Biosystems, Courtaboeuf, France). Total DNA from 22 strains of *X. axonopodis* pv. *allii* isolated from various geographical locations and six other xanthomonads were used (see Tables S1 and S2 in the supplemental material). PCR amplification, extraction of fragments from agarose gels, and cloning were performed under conditions described elsewhere (38). Sequence data were obtained for *X. axonopodis* pv. *allii* strains CFBP 6364, CFBP 6366, CFBP 6369, and CFBP 6379 using the double-strand single-pass sequence method (Genome Express, Meylan, France) and a nucleotide collection (nr/nt) available online (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi; accessed November 2009). In all experiments, sequence alignment and sequence analysis were performed using the Geneious software (v4.7, Biomatters Ltd., Auckland, New Zealand).

**Selection of AFLP markers.** Preamplification and selective amplification of amplified fragment length polymorphism (AFLP) sequences were performed with a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Courtaboeuf, France). Preliminary data (33; I. Robine-Soustrade, unpublished data) allowed identification of putative specific markers of *X. axonopodis* pv. *allii*. In the present study, *X. axonopodis* pv. *allii* strains CFBP 6369, CFBP 6380, CFBP 6384, JX36-1, CFBP 6386, and CFBP 6382 represent different pathovars of *X. axonopodis* pv. *allii* strains, which did not produce amplicons using the RAPD 80-21 primer, were used. One strain of *X. citri* pv. citri (IAPAR 306) and one strain of *X. campestris* pv. campestris (CFBP 5251) were used as controls. AFLP experiments were performed as described previously (2), except that unlabeled MspI + A, MspI + C, MspI + T, or MspI + G primer and the SacI + C or SacI + CT primer were used for selective amplification. All adapters and AFLP primers (Applied Biosystems, Courtaboeuf, France) used are listed in Table 1. After migration on 2% agarose gels as described elsewhere (37), DNA was visualized using a silver staining method (34). Potentially interesting AFLP fragments were removed from agarose gels using a scalpel and incubated at 60°C for 2 h in 50 µl 10× Goldstar Red Tq polymerase buffer (Eurogentec, Seraing, Belgium). Five microliters was used as the template for amplification with the corresponding unlabeled selective MspI + N/SacI + CT primer pair under selective amplification conditions, as mentioned above. The DNA fragments were cloned and sequenced as described above. The F1154 and R1391 primers (Table 1) were designed using either the sequence of the 238-bp amplicon or the 60-nt gene sequence of *avrRxv* (21), and bacterial suspensions from cultures (a single colony in 1 ml of sterile deionized water, boiled for 2 min and chilled on ice), and seed extracts prepared as described below.

Specific primers were designed with Primer3 software (44) and were synthe-
PCR-BASED ASSAY TO DETECT XANTHOMONAS IN ONION SEEDS

FIG. 1. Amplification of a DNA fragment similar to theavrRxv gene in X. axonopodis pv. allii. The arrows indicate the positions and directions of priming of the primers chosen in the X. axonopodis pv. vesicatoriaavrRxv gene and the X. axonopodis pv. allii AFLP fragment.

Sized by Genecust (Evry, France). The sequences of the primers used for the first and second rounds of amplification are shown in Table 1. The first round of PCR was performed using 25-μl reaction mixtures containing 3 mM MgCl₂, 100 μM each dNTP, 0.2 μM primer Pxaa1U, 0.2 μM primer Pxaa1L, 0.2 μM primer Pxa2U, 0.2 μM primer Pxa2L, 1.25 μL Goldstar Red Taq polymerase (Eurogentec, Seraing, Belgium), and 0.5 to 5 μl template DNA (see below) in 75 mM Tris-HCl–20 mM (NE4)₅SO₄–0.01% Tween 20 buffer (pH 8.8). The amplification program included denaturation at 94°C for 5 min, 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 2 min, and a final extension step at 72°C for 5 min. The second round of PCR was performed with 1 to 5 μl of amplicons obtained from the first DNA reaction (see below) in 25-μl mixtures as described above for the first round, except that 0.2 μM primer Pxa1U, 0.2 μM primer Pxa1L, 0.2 μM primer Pxa2U, and 0.2 μM primer Pxa2L were used. The amplification program consisted of denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in 1% (amplicons from the first-round PCR) or 3% (amplicons from the nested PCR) Seakem LE agarose (FMC Bioproducts, Philadelphia, PA) and stained with ethidium bromide.

Specificity and sensitivity of multiplex nested PCR. DNA fragments amplified with the Pxaa1U/Pxa2L primer pair were sequenced for 27 X. axonopodis pv. allii strains (see Table S1 in the supplemental material) and 18 strains identified as members of other Xanthomonas pathovars (see Table S2 in the supplemental material). The fragments amplified with the Pxa2U and Pxa2L primers were sequenced for 28 X. axonopodis pv. allii strains (see Table S1 in the supplemental material) and 17 strains identified as members of other Xanthomonas pathovars (see Table S2 in the supplemental material). For these experiments, PCRs were performed as described above, except that Dap Goldstar polymerase (Eurogentec, Seraing, Belgium) was used, as described above, with bacterial suspensions as the templates. Amplicons were sequenced using Macrogen (Seoul, South Korea), as described previously. All strains listed in Tables S1 and S2 in the supplemental material were tested with the multiplex nested PCR assay (see above) using 0.5-μl bacterial suspensions as the templates. Amplicons were diluted 1:100 in deionized water for the second round of PCR to prevent inhibition of the PCR due to high template DNA concentrations.

The sensitivity of the multiplex nested PCR was determined using bacterial dilution series, one-half of which were mixed with seed samples. Suspensions prepared from overnight cultures on YPGA plates of X. axonopodis pv. allii strains CFBP 6385, CFBP 6366, and CFBP 6367 were adjusted spectrophotometrically to obtain a concentration of 1 × 10⁶ CFU ml⁻¹ (optical density at 600 nm [OD₆₀₀] = 0.05) and serially 10-fold diluted in 0.01 M sterile Sigma 7-9 buffer (pH 7.2; Sigma, Saint-Quentin Fallavier, France). Samples containing 10 g of healthy onion seeds (A. cepa cv. Véronique) harvested from plants growing in an area free of X. axonopodis pv. allii were soaked in 50 ml of 0.01 M sterile Sigma 7-9 buffer (pH 7.2) and inoculated with bacterial suspensions at final concentrations ranging from 1 × 10⁴ CFU ml⁻¹ to 1 × 10⁵ CFU ml⁻¹. Negative controls were inoculated with 0.01 M sterile Sigma 7-9 buffer (pH 7.2). Similar dilution series not mixed with seed samples were analyzed concomitantly. After 48 h of maceration at 4°C, samples were plated in duplicate on NCTM1 semiselective medium (42) with a spiral device (Interscience, Saint Nom La Bretêche, France), and bacterial genomic DNA was extracted using a quick alkaline DNA extraction method (5). For each experiment, two (for suspensions containing ≥1 × 10⁷ CFU ml⁻¹) or three (for suspensions containing <1 × 10⁴ CFU ml⁻¹) aliquots (5 μl each) were used as templates. The experiment was replicated once.

Detection of X. axonopodis pv. allii in seed samples collected from a diseased field. A seed lot collected from onion plants (A. cepa cv. Véronique) growing in experimental diseased plot P2 (15) was checked for the presence of X. axonopodis pv. allii. The number of samples (35 samples per experiment, each consisting of 10 g of seeds) was determined based on the hypergeometric distribution in order to detect at least one contaminated seed in 30,000 seeds (P = 0.05). The experiment was replicated once. Seed samples were soaked in 50 ml of 0.01 M sterile Sigma 7-9 buffer (pH 7.2; Sigma, Saint-Quentin Fallavier, France) for 48 h. Undiluted macerates and macerates diluted 1:10 were plated on the semi-selective NCTM1 medium with a spiral device (Interscience, Saint Nom La Bretêche, France). All macerates were analyzed at least twice by multiplex nested PCR after alkaline DNA extraction, as described above. Negative controls in which water was used as the template were included in all experiments. Nested PCR is sometimes known to produce false-positive results. Therefore, only samples in which the expected DNA fragments were detected at least twice were scored as positive. Multiplex nested PCR was also used to confirm the identity of xanthomonad-like colonies recovered from seed macerates on the semiselective medium. Seed contamination rates derived from plate counts and from multiplex nested PCR analyses were calculated as described elsewhere (22). The same seed lot was mixed with healthy onion seeds (A. cepa cv. Véronique) at ratios of 1:1.1, 1:2.0, and 1:12.8, and two, two, and three independent samples, respectively, from these mixtures were analyzed as described above.

The exact distribution of positive samples in 35 samples for a contamination rate of 0.01% was calculated using Bayes’ formula and the binomial distribution of the number of positive seeds. The correlation between the contamination rates calculated from plate counts or in multiplex nested PCR assays and the dilution ratios of infected seeds to healthy seeds was analyzed with the Pearson’s product moment correlation coefficient (32). All statistical analyses were performed using R statistical software (version 2.8.1; R Development Core Team, Vienna, Austria).

Pathogenicity tests. The pathogenicity of all strains of X. axonopodis pv. allii (see Table S1 in the supplemental material) was confirmed by verifying Koch’s postulates using the host species from which the strain originated. The pathogenicity of some genetically related strains not assigned to this pathovar (see Table S2 in the supplemental material) was checked using onion cultivar Red Creole as described previously (42).

Nucleotide sequence accession numbers. The DNA sequences of the AFLP-derived fragment (1,948 bp) and the 80-21 RAPD fragment (987 bp), both obtained from strain CFBP6366, have been deposited in the GenBank database under accession numbers GU084403 and GU084404, respectively. The GenBank accessions for the DNA sequences amplified with primers Pxa1U and Pxa1L and with primers Pxa2U and Pxa2L are GU736580 to GU736624 and GU736625 to GU736669, respectively.

RESULTS

Selection of RAPD and AFLP markers specific for X. axonopodis pv. allii. None of the RAPD markers tested were present in all strains of X. axonopodis pv. allii, nor were they present in strains classified as members of other pathovars. Nevertheless, several fragments were amplified from most X. axonopodis pv. allii strains and were not amplified from any unrelated bacterial strains except X. axonopodis pv. vesicatoria strains. The 80-21 primer (5′ACGCCGCAGG) produced an approximately 940-bp amplicon for 70% of the X. axonopodis pv. allii strains tested. DNA sequences derived from this fragment from strains CFBP 6364, CFBP 6366, CFBP 6369, and CFBP 6379 were highly similar (99% identity over 937 bp).

Comparison of the target amplified sequence to sequences present in strains classified as members of other pathovars. The 80-21 primer (5′ACGCCGCAGG) produced an approximately 940-bp amplicon for 70% of the X. axonopodis pv. allii strains tested. DNA sequences derived from this fragment from strains CFBP 6364, CFBP 6366, CFBP 6369, and CFBP 6379 were highly similar (99% identity over 937 bp). Comparison of the target amplified sequence to sequences stored in the GenBank database (BLASTN) revealed a high level of similarity (98% over 933 bp) with two portions of contiguous genes (831 bp and 102 bp) encoding the type IV pilus assembly proteins PilW and PilX of X. axonopodis pv. vesicatoria, respectively. Other RAPD markers were analyzed in the same way, although no other RAPD marker was shared by the strains not amplified when the 80-21 primer was used.

Two AFLP fragments present in a majority of X. axonopodis pv. allii strains, including the strains for which no amplicons were obtained with the 80-21 RAPD fragment, were identified. Due to the complexity of the AFLP profiles obtained when two selective bases were used in the selective amplification step,
only simplified AFLP profiles using three selective bases were used to recover the target DNA fragments from gels. Even under these more stringent conditions, DNA sequences ligated into pGEM vectors suggested that contamination during excision of the fragments or comigration of amplification products that were the same size may have occurred. Interestingly, a clone obtained in the AFLP analysis using three selective nucleotides was obtained from strain CFBP 6382. This clone had a 270-bp sequence showing 90% similarity to a portion of the avrRpv avirulence gene of X. axonopodis pv. vesicatoria (GenBank accession number L20423). This fragment was amplified in all strains analyzed (88 to 100% identity with avrRpv for 238-bp amplicons depending on the strain). Primers used for PCR amplification and sequencing were designed using the combined chimeric sequence of this fragment and the sequence of avrRpv from X. axonopodis pv. vesicatoria. Using these primers, DNA sequences covering 1,948 bp were obtained for X. axonopodis pv. allii strains CFBP6357, CFBP 6366, CFBP 6382, and CFBP 6386 and DNA sequences covering 1,950 bp were obtained for strains CFBP 6384 and JX36-1. For strain CFBP 6380, a 1,504-bp partial sequence was obtained. All these sequences matched the avrRpv sequence from X. axonopodis pv. vesicatoria with levels of similarity ranging from 87 to 99%.

**Specificity and sensitivity of the multiplex nested PCR assay.** A multiplex nested PCR assay based on the two described DNA markers (referred to below as PIL and AVR) was performed by selecting compatible external and internal primers from the most conserved regions of sequences recovered for X. axonopodis pv. allii. The theoretical sizes of AVR amplicons were 995 bp (first round) and 401 bp (nested round) for all sequences analyzed. For the PIL marker, theoretical sizes of the amplicons were 697 bp (first round) and 447 bp (nested round) for all sequences analyzed except the strain CFBP 6364 sequence, which had a 3-bp deletion (694-bp and 444-bp amplicons). When the multiplex PCR assay was performed with all X. axonopodis pv. allii strains (n = 86), three categories of responses were observed on gels after the first round of amplification. Two amplicons of the expected sizes, about 700 bp (corresponding to the PIL marker) and 990 bp (corresponding to the AVR marker), were obtained for all strains from Cuba (n = 2), Japan (n = 2), Mauritius (n = 15), Réunion Island (n = 17), and Hawaii (n = 13) and for four strains from Brazil. One 700-bp amplicon (PIL) was amplified from all strains from Georgia (n = 3) and Venezuela (n = 7), from four strains from Colorado, and from two strains from South Africa. One 990-bp amplicon (AVR) was observed for all strains from Barbados (n = 5) and Texas (n = 7), three strains from South Africa, one strain from Brazil, and one strain from Colorado. In the second round of PCR, the corresponding internal amplicons were observed for the different strains, and they included two DNA fragments that were approximately 450 bp (PIL) and 400 bp (AVR) long for the first group of strains and single 450-bp and 400-bp amplicons for the second and third groups of strains, respectively (Fig. 2; see Table S1 in the supplemental material).

No amplification product was observed when the multiplex PCR assay was performed for saprophytic strains isolated from onion or for bacteria belonging to other bacterial genera (see Table S2 in the supplemental material). Most Xanthomonas strains also did not produce amplicons, except for a few strains belonging to X. axonopodis genetic group 9.2 sensu Rademaker et al., X. axonopodis pv. vesicatoria, X. axonopodis pv. citrulnemo, X. axonopodis pv. cassavae, X. axonopodis pv. desmodii, X. axonopodis pv. desmodiagencytici, X. axonopodis pv. phyllanthi, X. axonopodis pv. tamarindii, and X. axonopodis pv. lespezeae. Depending on the pathovar, one or both approximately 700-bp and 990-bp DNA fragments (and the corresponding nested 450-bp and 400-bp amplicons) were observed. Amplicons were also observed for the AVR marker for strains of X. axonopodis pv. begoniae (genetic group 9.1 sensu Rademaker et al.). Pathogenicity tests showed that none of the strains positive for one or both markers in the multiplex assay were pathogenic to onion. Sequencing of the amplicons from the first round of PCR confirmed the theoretical sizes of nested amplicons and showed that for the PIL marker the same 3-bp deletion occurred in the strains that originated from Cuba (n = 2) and Japan (n = 2) and in all of the strains belonging to X. axonopodis pv. vesicatoria. For strain LMG 955 a 3-bp insertion was found at the same site (450-bp nested amplicon). When these sequences were compared, specific signature sequences permitted us to distinguish X. axonopodis pv. allii from all nontarget strains except X. axonopodis pv. lespezeae strain NCPPB 938 (see the GenBank sequences deposited under accession numbers GU736580 to GU736624 and GU736625 to GU736669). Enzymatic restriction signatures that allowed us to distinguish X. axonopodis pv. allii from X. axonopodis pv. vesicatoria and X. axonopodis pv. begoniae were found (data not shown).

The first round of PCR performed with dilution series of strains CFBP 6366, CFBP 6367, and CFBP 6385 mixed with seed samples resulted in a detection sensitivity of 1 × 10^4 CFU ml^-1, as determined by dilution plating on YPGA and NCTM1 medium. When multiplex nested PCR was used, the detection limit was 1 × 10^2 CFU ml^-1. A signal was often obtained (for one or two of three replicates) when suspensions containing 1 × 10^2 CFU ml^-1 were used as templates. The sensitivity of the assay was the same when the same dilution series that were not mixed with seed samples were tested.

**Detection of X. axonopodis pv. allii in seed samples collected from a diseased field.** X. axonopodis pv. allii was detected in both experiments using the multiplex nested PCR assay with six and nine positive samples (Table 2). The values obtained corresponded to an average contamination rate (CR) of
0.01%. When calculating the exact distribution of positive samples for 35 samples for a CR of 0.01%, we found dispersion between 3 and 11 positive samples at a probability level of 96.6% and dispersion between 4 and 10 positive samples at a probability level of 89.6%, and the mean number of positive lots was 6.76. These data were consistent with the observed values for positive samples. Meanwhile, the pathogen was recovered on semiselective NCTM1 medium from four and one samples with an average CR of 0.0031%. Recovery of target bacterial colonies on NCTM1 medium proved to be difficult because of high population densities of saprophytic bacteria (>1 × 10^6 CFU per g of seeds) that were associated with onion seeds and rapidly colonized agar plates. No amplicon was obtained and no colony of X. axonopodis pv. allii was isolated on NCTM1 medium for two healthy seed lots analyzed as negative controls. The assay that was developed accurately estimated seed contamination rates, and there was a positive correlation (r = 0.90) between “theoretical” (as determined by seed dilution ratios) and experimentally determined seed contamination rates calculated from PCR data. In contrast, such a correlation could not be demonstrated when contamination rates derived from plate counts were used. At an experimentally determined contamination rate of 0.0037%, corresponding to one infected seed in 27,340 seeds, the multiplex nested PCR assay allowed detection of the bacterium in each nested PCR replicate. At a higher dilution (CR, 0.0012%; i.e., one contaminated seed in 82,000 seeds), only one of three replicates was positive.

**DISCUSSION**

A multiplex nested PCR was developed for specific and sensitive detection of X. axonopodis pv. allii from onion seeds. Due to the high genetic diversity of this pathogen (11, 12, 33, 41), two PCR-based sequence-characterized amplified regions (SCARs) identified in RAPD and AFLP analyses were required to detect all isolates of this pathovar. The RAPD technique has been widely used for SCAR development to obtain PCR diagnostic tools for bacterial plant pathogens (23, 38, 54). Examples of SCARs derived from AFLP analysis are less common in molecular phytodiagnostics (10, 36), whereas this technique is capable of generating a large number of markers over a relatively short time without any prior sequence knowledge (48). In order to convert AFLP markers into SCAR markers, we reduced the complexity of AFLP fingerprints by increasing the number of selective bases. In spite of this precaution, the recovered DNAs were heterogeneous in terms of fragment composition (data not shown), which was probably the result of coisolation of amplification products that were of similar sizes, a situation previously documented for AFLP analysis (7). Nevertheless, here we succeeded in isolating an AFLP fragment of interest.

The target sequence identified by RAPD-PCR analyses displayed a high level of nucleotide similarity with sequences encoding the PilW and PilX proteins of X. axonopodis pv. vesicatoria. These proteins are involved in assembly of type IV pili (fimbriae), as demonstrated for Pseudomonas aeruginosa (3). Fimbriae are present in plant-pathogenic bacteria. They are involved in host colonization, plant cell adhesion, cell aggregate formation, or twitching motility. They are, therefore, important components of plant-bacterium interactions (9, 18, 19, 30). The target sequence identified in the AFLP analysis showed a high level of nucleotide similarity with the avrRxv avirulence gene of X. axonopodis pv. vesicatoria (57). This gene encodes a type III effector protein belonging to the AvrRxv/YopJ (C55) family of proteins, which presumably function as proteases and modulate the host response (14, 26). Thus, the two regions targeted in our multiplex PCR assay are potentially involved in pathogenic interactions with host plants.

**TABLE 2. Contamination rates and numbers of positive samples detected using seed macerates of infected seed lots**

<table>
<thead>
<tr>
<th>Seeds</th>
<th>Assay</th>
<th>No. of seed samples</th>
<th>Multiplex nested PCR detection</th>
<th>Semiselective isolation</th>
<th>No. of lots detected by both methods</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CR (%)</td>
<td>No. of positive lots</td>
<td>CR (%)</td>
</tr>
<tr>
<td>Seed lot P2 (CR, 1/10,000)*</td>
<td>Assay 1</td>
<td>35</td>
<td>0.000077</td>
<td>6</td>
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<tr>
<td></td>
<td>Assay 2</td>
<td>35</td>
<td>0.000121</td>
<td>9</td>
<td>0.000012</td>
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<tr>
<td></td>
<td>Mean</td>
<td></td>
<td>0.000099</td>
<td></td>
<td>0.000031</td>
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<tr>
<td>Seed lot P2 mixed with healthy seeds (1:1.1) (CR, 1/21,000)*</td>
<td>Assay 1</td>
<td>35</td>
<td>0.000050</td>
<td>4</td>
<td>0.000000</td>
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<tr>
<td></td>
<td>Assay 2</td>
<td>35</td>
<td>0.000037</td>
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<tr>
<td></td>
<td>Mean</td>
<td></td>
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<td></td>
<td>0.000012</td>
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<tr>
<td>Seed lot P2 mixed with healthy seeds (1:2) (CR, 1/30,000)*</td>
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<td>35</td>
<td>0.000037</td>
<td>3</td>
<td>0.000000</td>
</tr>
<tr>
<td></td>
<td>Assay 2</td>
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<td>0.000037</td>
<td>3</td>
<td>0.000012</td>
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<tr>
<td></td>
<td>Mean</td>
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<td></td>
<td>0.000006</td>
</tr>
<tr>
<td>Seed lot P2 mixed with healthy seeds (1:12.8) (CR, 1/138,000)*</td>
<td>Assay 1</td>
<td>35</td>
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<td>0</td>
<td>0.000012</td>
</tr>
<tr>
<td></td>
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<td>3</td>
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</tr>
<tr>
<td></td>
<td>Mean</td>
<td></td>
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<tr>
<td>Healthy seeds</td>
<td>Assay 1</td>
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</tr>
<tr>
<td></td>
<td>Assay 2</td>
<td>35</td>
<td>0.000000</td>
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<td>0.000000</td>
</tr>
</tbody>
</table>

* The seed contamination rate was determined by multiplex nested PCR analyses.

* The theoretical seed contamination rate was obtained by diluting seed lot P2 with healthy seeds.
The internal primers of the multiplex PCR assay directed amplification for all strains of X. axonopodis pv. allii. Depending on the strain, one or two fragments were amplified. No amplification product was obtained for taxonomically unrelated bacteria (41 strains) or for saprophytic bacteria commonly isolated from onion leaves and seeds (14 strains). Amplicons were also obtained for some strains that are not classified in X. axonopodis pv. allii but belong to X. axonopodis genetic subgroup 9.1 or 9.2 _sensu_ Rademaker et al. (35). Some of the pathogens are well documented as they cause diseases of economic concern (e.g., _X. axonopodis_ pv. vesicatoria, _X. axonopodis_ pv. citrulmo, _X. axonopodis_ pv. cassavae, and _X. axonopodis_ pv. begoniae), whereas no data or little data are available for the other pathogens. There have been no reports of detection of _Xanthomonas_ pathogens other than _X. axonopodis_ pv. allii in onion seeds. The probability of finding these pathogens on onion plants or seeds is negligible because the capacity to induce symptoms on onion leaves is specific to _X. axonopodis_ pv. allii strains, as demonstrated by inoculation of onion plants with different pathogens belonging to group 9.2 (11). In this study, we also verified that all strains not classified as _X. axonopodis_ pv. allii that displayed a positive PCR signal were not pathogenic to onion. As these bacteria cannot multiply over a long period of time if they are in contact with onion tissue, it is very unlikely that they would be detected on onion plants and consequently in seeds. Thus, this molecular tool can be routinely used to specifically evaluate rates of seed contamination by _X. axonopodis_ pv. allii. Nevertheless, the presence of signature sequences for most of the strains can be used to differentiate the other pathogens from _X. axonopodis_ pv. allii if a false-positive signal is suspected. If necessary, AFLP or multilocus sequence analysis methods can also be used to confirm the diagnosis after isolation of the bacteria (8).

The PCR-based seed assay detected _X. axonopodis_ pv. allii in artificially inoculated seed samples with contamination levels ranging from $5 \times 10^2$ to $5 \times 10^7$ CFU g$^{-1}$. It is likely that the alkaline extraction procedure that was used prior to PCR concentrated the DNA and neutralized PCR inhibitors in onion seed extracts, as suggested by preliminary experiments (data not shown). The rate of contamination of the seed lot was estimated to be 0.01% by the multiplex nested PCR assay, consistent with previous data (15, 42). This rate is similar to those reported previously for other pathosystems involving _xanthomonads_ (1). The sensitivity threshold of the multiplex nested PCR assay was found to be 1/27,340 when it was determined with the same naturally contaminated seed lot mixed with healthy seeds. This level of sensitivity is greater than the contamination rate reported for BBO outbreaks in a tropical environment (4.5/10,000) (43). This threshold is also consistent with the tolerance standards currently recommended in the International Rules of Seed Health Testing for phytopathogenic bacteria and with a lower threshold recently defined for _X. campestris_ pv. campestris for avoiding spread among transplants in seed trays (39).

Our new multiplex nested PCR assay is less labor-intensive and time-consuming than isolation on semiselective media. The latter technique requires further evaluation of suspect colonies using other identification methods, including serological or molecular techniques and/or pathogenicity tests.

When used with naturally infected seed samples, the PCR assay was found to detect more positive samples than the traditional plating method. This was often due to high populations of microbiota present on seeds, which were able to grow on the semiselective medium and inhibited the development of the target bacterium (42). Alternatively, it is possible that our PCR-based method detects free DNA from nonviable bacteria, as demonstrated for PCR detection of _X. campestris_ pv. carotae (23), and thus results in false-positive responses. Nonetheless, detection of free target DNA in a seed lot represents a history of contamination, which indicates that there is a need for further analysis. We plan to compare the performance of biological enrichment followed by PCR (BIO-PCR) (49) and ethidium monooazide PCR (EMA-PCR) (27, 45) in terms of their capacities to determine the rates of occurrence of viable target cells in seed samples. We concluded that the multiplex nested PCR assay described here is a reliable and sensitive procedure for detecting and identifying _X. axonopodis_ pv. allii, the BBO pathogen, in onion seeds and that it should be useful for international sanitary surveillance of seed exchanges. Additionally, this molecular tool could be useful for ecological and epidemiological investigations, especially investigations to determine the relationship between seed contamination and disease incidence or to understand better the life cycle of _X. axonopodis_ pv. allii and assess the relative importance of the different stages of dissemination and conservation of this pathogen. A French patent concerning this detection technique and the target sequences is pending.

ACKNOWLEDGMENTS

This work was funded by grants from the European Union (FEOGA), the Conseil Régional de La Réunion, and CIRAD. We thank Philippe Roumagnac, Michel Roux-Cuvelier, and Emmanuel Jouen for helpful discussions; Christian Vernière and Philippe Chiroleu, and Walter Grondin for technical assistance.

REFERENCES


