A Rapid and Sensitive PCR-Based Assay for Concurrent Detection of Bacteria Causing Common and Halo Blights in Bean Seed

Patrice Audy, Carol E. Braat, Gilles Saindon, Henry C. Huang, and André Laroche

Research Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta T1J 4B1.

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ABSTRACT

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A quick polymerase chain reaction (PCR)-based procedure was developed for concurrent detection of the causal agents of bean common blight, *Xanthomonas campestris* pv. *phaseoli*, and bean halo blight, *Pseudomonas syringae* pv. *phaseolicola*, in bean seed. A rapid DNA extraction procedure, consisting of briefly wetting intact or crushed seeds with a solution of sodium hydroxide, was used to extract bacterial DNA from externally and internally blight-contaminated seeds. G+C-rich oligonucleotide primers were designed from the phaseolotoxin gene cluster of *P. syringae* pv. *phaseolicola* and tested under high-stringency conditions in PCR assays. The HB14 primers specifically directed the amplification of a 1.4-kb fragment from DNA of 19 *P. syringae* pv. *phaseolicola* isolates, whereas templates from 62 other bacterial strains, including the bean pathogens *P. syringae* pv. *syringae* and *X. campestris* pv. *phaseoli*, and plant-pathogenic species of *Agrobacterium*, *Clavibacter*, *Erwinia*, and *Xanthomonas* did not produce any discrete bands upon amplification. The HB14 primers for *P. syringae* pv. *phaseolicola,* and the previously reported G+C-rich X4 primers, specific for *X. campestris* pv. *phaseoli*, amplified discrete DNA fragments by soaking extracts of white bean seeds contaminated with halo and common blights, respectively. DNA extracts from blight-infested colored seeds were recalcitrant to PCR-amplification unless polyvinylpyrrolidone was added to the extraction buffer. In combination, X4 and HB14 primers successfully detected individual and mixed infections of bean common and halo blights and yielded distinctive DNA fragments from batches containing as few as 1 infected seed in 10,000 seeds.

Additional keywords: diagnosis, *Phaseolus vulgaris*.

Common and halo blights, caused by *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola*, respectively, are major seedborne diseases of bean (*Phaseolus vulgaris* L.) worldwide. *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* have tremendous disease potential, because a few infected seeds are sufficient to initiate a general epidemic under favorable conditions (25,29). Economic losses due to bean blights result from seed yield reductions and loss of seed marketability (17,22). Both bacteria can survive and grow epiphytically on tolerant bean genotypes without causing symptoms (3,24). Survival of *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* in bean debris from previous crops has been documented, but its importance in disease epidemiology is still unclear (6,8,12). Seedborne contamination, whether internal or external, is the most important means of survival for *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* and is the primary source for their local and widespread dissemination (17,22). The use of pathogen-free seeds, cultural practices and, when available, resistant cultivars, are the only practical methods of controlling common and halo blights in bean.

The success of bean seed-certification programs depends on accurate tests for detecting pathogenic seedborne bacteria. Many countries have established strict quarantine regulations for bean blights, based on field inspection and laboratory analysis, with a zero level of tolerance for some classes of pedigreed seeds (15,30,32). The current seed-testing procedures, which consist of plant inoculation techniques (32) and plating seed-soak extracts on differential and semiselective media (23,27), are time-consuming and labor intensive. Semiselective media favor isolation of bean-blight bacteria from saprophytic species but are not sufficiently selective to detect all strains of the target organisms (28) nor distinguish between the closely related bean pathogen *P. syringae* pv. *syringae* (15). Serological assays are not yet sufficiently specific or sensitive to be used routinely (23,27). Therefore, there is a pressing need for rapid, specific, and highly sensitive diagnostic assays for bean common and halo blights.

Nucleic acid hybridization probes consisting of DNA sequences unique to *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* have provided highly specific tools for detection and identification of bean blight pathogens (7,20). DNA hybridization probes were effective with large numbers of target bacterial cells, but their sensitivity was impaired at low pathogen populations in the presence of a large excess of heterologous DNA or RNA. Using oligonucleotide primers designed from the sequences of the DNA hybridization probes specific to *X. campestris* pv. *phaseoli* (7) and *P. syringae* pv. *phaseolicola* (20), polymerase chain reaction (PCR)-amplification assays have been developed for detection of bean common (1) and halo blights (15,21). In bean seeds naturally infected with *P. syringae* pv. *phaseolicola*, the bacterium was detected in commercial seed lots where conventional methods failed to yield positive readings (15). However, the method required time-consuming steps for the extraction of bacterial DNA and cost-adding Southern blots to achieve high sensitivity (15). A

Corresponding author: G. Saindon; E-mail address: saindon@abrsle.agr.ca

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combined use of plating media and two consecutive PCR runs allowed sensitive detection of *P. syringae* pv. *phaseolicola* live cells, but the method (BIO-PCR) still required at least 4 days (21). Audy et al. (1) developed a sensitive 1-day test for detection of *X. campestris* pv. *phaseoli* from common-blight lesions on bean leaves that uses a quick alkaline DNA extraction method and specific G+C-rich primers in a two-step PCR protocol. In this study, we describe the development of a rapid and sensitive PCR assay for simultaneous detection of seedborne *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* in large samples of bean seeds.

TABLE 1. Bacterial strains used

Strain	Isolates	Location	Source
Pseudomonas syringae pvs.			
apii	Psap-1	New York	J. Norelli
aptata	Psapt-1	New York	J. Norelli
coronafaciens	Pscr-2, Pscr-4	New York	J. Norelli
glycinea	Psgly-2, Psgly-3	New York	J. Norelli
lachrymans	Pslach-1	New York	J. Norelli
phaseolicola	HB9, HB33, HB93, PS90SC-5	Alberta	G. Saindon
	HBA1, HBA2	Alberta	P. Audy
	Psph-3, Psph-8	New York	J. Hunter
	B45, B132, B297, B301, K196, P193, 8318	Colorado	H. F. Schwartz
	ATCC 11355, ATCC 11365, ATCC 19304, ATCC 21781		ATCC
pisi	Pspi-6	New York	J. Norelli
sesami	Psses-1	New York	J. Norelli
syringae	PA1, PA2, PA3, PA4, PA5, PA6	Alberta	P. Audy
	Ps-30, Ps-SD438	New York	R. Dickey
	190-38, 211-50-1	Oregon	M. Powelson
	B107, B133, B296, F84-24, K207, P206	Colorado	H. F. Schwartz
tomato	DC 3000	Ontario	D. Cuppels
Xanthomonas campestris pv.			
phaseoli	EK11, Xp816	Nebraska	M. Schuster
	ATCC 9563, ATCC 10199		ATCC
	ATCC 17801		ATCC
phaseoli var. *fuscans*	Bxp12, Xp fusc wallen	Ontario	B. N. Dhanvantari
	ATCC 19315		ATCC
	Mal6	Malawi	R. L. Gilbertson
alfalfae	XA1	Kansas	D. L. Stuteville
armoraciae	275	Wisconsin	D. K. Willis
carotae	Xcar1	California	R. L. Gilbertson
campestris	Cp670	Québec	M. Lacroix
	Xcc295	Wisconsin	R. L. Gilbertson
holcicola	XCH429	Kansas	J. L. Leach
juglandis	252	California	R. L. Gilbertson
pelargonii	ATCC 8721		ATCC
	Pg730	Québec	M. Lacroix
pruni	ATCC 10016		ATCC
vesicatoria	ATCC 11633		ATCC
	Vs715	Québec	M. Lacroix
vitians	93-00	California	R. L. Gilbertson
X. fragariae	Fg38	Québec	M. Lacroix
X. oryzae pv. *oryzae*	XO61	Philippines	J. L. Leach
X. saprophytes (bean)	5, D21, P3L3, 232	Wisconsin	R. L. Gilbertson
	Xb1, Xb2, Xb4, Xb5	Alberta	P. Audy
Agrobacterium tumefaciens	LBA 4404	Netherlands	R. A. Schilperoort
Clavibacter michiganense subsp. *sepedonicus*	RR2	Alberta	H. C. Huang
Erwinia carotovora subsp. *atroseptica*	BL3	Alberta	H. C. Huang
E. herbicola	EH-11	Wisconsin	R. L. Gilbertson

MATERIALS AND METHODS

Bacterial strains and media. All bacterial cultures (Table 1) were freshly prepared from stocks stored in 7% (vol/vol) dimethyl sulfoxide at –80°C (2). *X. campestris* pv. *phaseoli* strains were maintained on semiselective MXP medium (5), and all other *Xanthomonas* strains were maintained on yeast-dextrose-$CaCO_3$ agar (33). *P. syringae* pv. *phaseolicola* strains were cultured on semiselective MSP (14), *P. syringae* pv. *syringae* were cultured on semiselective KBC (14), and all other *P. syringae* strains were cultured on KB agar (11). All other bacteria were cultured on medium 523 (10).

Seed materials. The following bean (*P. vulgaris*) seed materials were used: (i) certified bean seed from Idaho (Foundation Seed Program of the University of Idaho, Moscow); (ii) seeds from plants produced in a controlled growth cabinet and artificially infected with *X. campestris* pv. *phaseoli* or *P. syringae* pv. *phaseolicola*; and (iii) four commercial seed lots grown in the southern Canadian prairies that were suspected of being contaminated with *X. campestris* pv. *phaseoli* or *P. syringae* pv. *phaseolicola*. Fifty seeds from each group were taken randomly and soaked at 4°C for 4 h in 20 ml of a sterile phosphate buffer: 0.05 M, pH 7.0, containing 0.85% NaCl and 0.01% Tween 20 (phosphate buffered saline [PBS]). The seed extracts were serially diluted and plated in triplicate onto MXP, MSP, and 523 media to determine the number of CFU of *X. campestris* pv. *phaseoli, P. syringae* pv. *phaseolicola,* and saprophytic bacteria, respectively. Each experiment was repeated twice. Certified bean seeds were free of common and halo blight pathogens and carried approximately 1×10^5 CFU of saprophytic bacteria per seed. Artificially blight-infected and commercial seeds had approximately 1×10^6 CFU per seed with a blight/saprophytic bacteria ratio of 1/50 and 1/100, respectively.

Artificially contaminated seeds were prepared from blight-free bean white-seeded cultivar US1140 (Great Northern) and color-seeded cultivars ISB82772 (kidney) and Othello (pinto), using common blight *X. campestris* pv. *phaseoli* strain EK11 and halo blight *P. syringae* pv. *phaseolicola* strain HB93 as inoculum. Bean plants were grown in 15-cm-diameter pots (two plants per pot) in a controlled growth cabinet (25°C, 16-h photoperiod) and inoculated 40 days after emergence, when the pods were half-filled. Blight bacterial strains were grown in shake culture at 26°C for 18 h in nutrient broth containing 0.25% glucose. Bacterial cells were pelleted at 10,000 × *g* for 30 s and resuspended in sterile 10 mM $MgSO_4$ just prior to inoculation (0.01 A_{600}, approximately 1×10^7 CFU/ml). Seeds were lightly punctured through the pods with a small needle (21G1, Becton Dickinson & Co., Rutherford, NJ) and injected with 10 to 20 µl of inoculum. Control seeds were mock-injected with a sterile 10 mM $MgSO_4$ solution. Plants were grown in the growth cabinet (25°C, 16-h photoperiod) for an additional 25 days before the seeds were harvested at maturity.

Sequencing and primer design for *P. syringae* pv. *phaseolicola*. PCR amplification of a 1.9-kb DNA fragment of the phaseolotoxin gene cluster of *P. syringae* pv. *phaseolicola* was directed by primers HM6 (5′-CGTGTCCTGGGATAAAAGC-3′) and HM13 (5′-GTTGAATTTCACTACCCG-3′) (15). The amplification was performed according to the conditions described by Prosen et al. (15) with *P. syringae* pv. *phaseolicola* genomic DNA (10 to 50 ng, strain HB93) extracted by an alkaline DNA extraction procedure (described below). PCR products were electrophoresed in a 0.8% low melting temperature agarose gel with Tris-acetate-EDTA buffer (2). The selected fragment was eluted and purified with a NaI glass-bead suspension (Geneclean II, Bio 101, La Jolla, CA) according to the manufacturer's recommendations. The fragment was partially sequenced by the dideoxynucleotide chain termination method (19) by a dye fluorescent sequencing method (Applied Biosystems, Foster City, CA) with *Taq* polymerase (Ampli*Taq*; Perkin-Elmer Cetus, Norwalk, CT). Paired primers HB14F (5′-CAACTCCGACACCAGCGACCGAGC-3′) and HB14R (5′-CC-

GGTCTGCTCGACATCGTGCCAC-3′) displaying similar thermal melting points, high G+C content, no stable hairpin and duplex structures, and similar thermal melting points, were designed with the software OLIGO, version 4.0 (National Biosciences, Plymouth, MN).

Extraction of bacterial genomic DNA. To assess the specificity of the HB14 primers, PCR amplifications were conducted with DNA extracted from pure bacterial cultures. Total bacterial genomic DNA was extracted by a quick alkaline DNA extraction method for plant material adapted from Wang et al. (31). Freshly grown single colonies on agar were used to start nutrient broth cultures. Cultures were grown at 26°C for 18 h, and cells from 1 ml of broth were used for DNA extraction. Bacterial broths were centrifuged (10,000 × *g*, 2 min), and cells were resuspended in 500 µl of 0.5 N NaOH. Five microliters of the lysate was transferred to a new tube containing 495 µl of 20 mM Tris-HCl, pH 8.0. One 5-µl aliquot was used per individual PCR assay. Water was used as a negative control. DNA yield was measured by the Hoechst dye assay method with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco) according to the manufacturer's instructions.

Extraction of bacterial genomic DNA from bean seeds was initially performed according to a protocol adapted from Prosen et al. (15). Individual seeds were soaked at 4°C for 16 h in 0.5 ml of sterile PBS. The seed extracts were centrifuged at 10,000 × *g* for 10 min, and pellets were dissolved in 100 µl of 0.5 N NaOH with or without 0.5% (wt/vol) PVP (polyvinylpyrrolidone; Sigma Chemical Company, St. Louis). Five microliters of the lysate was transferred to a 1.5-ml tube containing 495 µl of 20 mM Tris-HCl, pH 8.0. One 5-µl aliquot was used per individual PCR assay. Extracts from blight-free certified seeds were used as negative controls.

Simpler procedures were developed to extract bacterial genomic DNA from bean seeds. To assess external contamination, seeds were soaked thoroughly for 2 min in a solution of 0.5 N NaOH and 0.5% (wt/vol) PVP, either individually (200 µl per seed in a 1.5-ml tube) or in batches of 100 to 1,666 seeds (15 µl per seed in a polypropylene bag; Bel-Art Products, Pequannock, NJ). Individual seeds were centrifuged at 500 × *g* for 2 min, and 5 µl of the extract was transferred to a 1.5-ml tube containing 495 µl of 20 mM Tris-HCl, pH 8.0. For batches of 100 to 1,666 seeds, centrifugation was performed with a high-capacity centrifuge (Beckman J6-HC, rotor JS-4.2; Beckman Instruments, Fullerton, CA). The maximum capacity for the bucket of the JS-4.2 rotor was approximately 2,000 seeds. Batches containing 5,000 and 10,000 seeds were divided into bags of 1,666 seeds (three and six bags, respectively). Extracts from each bag were pooled, and 5 µl was transferred to a 1.5-ml tube containing 495 µl of 20 mM Tris-HCl, pH 8.0. One 5-µl aliquot was used per individual PCR assay. To detect internal contamination exclusively, *X. campestris* pv. *phaseoli*-contaminated seeds were washed in a solution of 0.5 N NaOH and 0.5% (wt/vol) PVP for 2 min and rinsed for 5 min, twice, in sterile PBS buffer. The seeds were blotted dry, placed individually in small polypropylene bags, and crushed coarsely with a hammer. The fragmented seed material was transferred to a 1.5-ml tube with 500 µl of 0.5 N NaOH and 0.5% (wt/vol) PVP and mixed thoroughly for 15 s by vortexing. The tube was briefly spun, and 5 µl of the extract was transferred to a 1.5-ml tube containing 495 µl of 20 mM Tris-HCl, pH 8.0. One 5-µl aliquot was used per individual PCR assay. Extracts from blight-free certified seeds were used as negative controls. All DNA samples (from seeds and bacteria) were used immediately in the PCR assays.

PCR assays. PCR-amplification assays were performed in a 25-µl reaction mixture containing 5 µl of DNA extract in 20 mM Tris-HCl, pH 8.0, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 µM each of upstream and downstream primers, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% [wt/vol] gelatin), and 1.25 units of *Taq* DNA polymerase (Perkin-Elmer). Mineral oil (molecular biology grade; Sigma) was added (25 µl) to prevent evaporation. For detection of *X. campestris* pv. *phaseoli*, primer X4e (5′-CGCCGGAAGCACGATCCT-CGAAG-3′) was paired with primer X4c (5′-GGCAACACCCG-ATCCCTAAACAGG-3′) (1). For detection of *P. syringae* pv. *phaseolicola*, primers HB14F (5′-CAACTCCGACACCAGCGA-CCGAGC-3′) and HB14R (5′-CCGGTCTGCTCGACATCGTG-CCAC-3′) were used. For concurrent detection of both pathogens, 0.25 µM each of X4c, X4e, HB14F, and HB14R were used simultaneously. A TwinBlock System thermocycler (Ericomp, San Diego, CA) was used for all PCR with the following thermal profile amplifications (unless otherwise indicated): initial denaturation at 95°C for 1 min with a manual "hot-start" step (4) at 80°C, followed by 35 repeated cycles of melting, annealing, and DNA extension at 95°C for 1 min, 65°C for 1 min, and 72°C for 2 min, respectively. For the last cycle, the extension time was increased to 10 min. In some experiments, annealing occurred at 60°C or was performed simultaneously with the DNA extension at 72°C for 2 min. The amplified DNA fragments were electrophoresed in 1.0% agarose gels in Tris-acetate-EDTA buffer (18) and visualized with ultraviolet light after ethidium bromide staining. Each experiment was repeated at least three times.

RESULTS

Priming under stringent conditions. Primers HM6 and HM13 directed the amplification of a 1.9-kb band from *P. syringae* pv. *phaseolicola* DNA when the annealing step occurred at 60°C (Fig. 1, lane A; 60°C). However, under otherwise identical conditions, priming was completely prevented at 65 and 72°C (Fig. 1, lanes A; 65 and 72°C). The G+C-rich HB14F and HB14R primers amplified a discrete DNA fragment of 1.4 kb from *P. syringae* pv. *phaseolicola* DNA at 60°C and also under the more stringent annealing conditions (Fig. 1, lanes B; 60, 65, and 72°C). Similar results were observed for the X4c and X4e primers, with the amplification of a single 730-bp DNA fragment from *X. campestris* pv. *phaseoli* DNA for this range of annealing conditions (Fig. 1, lanes C; 60, 65, and 72°C). The three thermal profiles for the HB14 and X4 primers apparently produced similar amounts of PCR products because the intensity of the ethidium bromide-

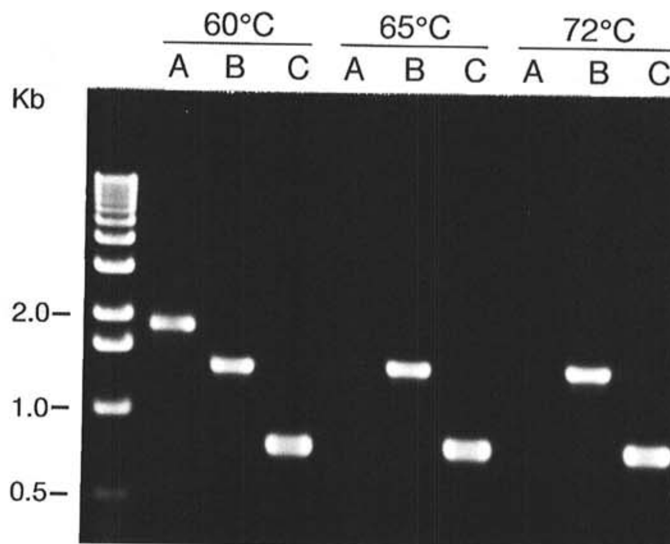

Fig. 1. Ethidium bromide-stained gel of polymerase chain reaction (PCR)-amplification products under different priming stringencies. Primer pairs HM6-HM13 (lanes A), HB14F-HB14R (lanes B), and X4c-X4e (lanes C) were used in a 35-cycle PCR run with an annealing step for 1 min at 60, 65, and 72°C. Template DNAs (20 ng) were from *Pseudomonas syringae* pv. *phaseolicola* cultivar HB93 (lanes A and B) and from *Xanthomonas campestris* pv. *phaseoli* cultivar EK11 (lanes C). Molecular weight standard (1.0-kb ladder; BRL) was run in the left lane (unmarked); numbers on the left indicate sizes in kilobases.

stained DNA fragments was comparable (Fig. 1, lanes B and C), indicating that priming was as efficient at 72°C as at 60°C for these G+C-rich primers.

Primer specificity for detection of *P. syringae* pv. *phaseolicola*. The G+C-rich HB14 primers exhibited a complete specificity for DNA extracted from halo blight bacteria by directing the amplification of the 1.4-kb DNA product from all 19 *P. syringae* pv. *phaseolicola* (Table 1) strains tested (data not shown). The DNA extracted from 62 other strains of bacteria (Table 1) that belonged to 9 pathovars of *P. syringae*, 11 pathovars of *X. campestris*, and isolates from species of *Agrobacterium*, *Clavibacter*, *Erwinia*, and *Xanthomonas* did not produce any discrete band on ethidium bromide-stained agarose gel upon amplification with the HB14 primers.

Detection of external seed contamination. Using the DNA extraction method involving a prolonged seed-soaking step, external contamination by *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* on artificially infected white cultivar US1140 seeds was consistently detected by PCR with the X4 and HB14 primers, respectively (Fig. 2, lane A). However, this procedure failed to yield distinctive PCR products from contaminated colored cultivars ISB82772 and Othello seeds (Fig. 2, lanes B and C). Similar results were observed when genomic DNA extracted from pure cultures of *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* were mixed with extracts from noncontaminated colored seeds of cultivars ISB82772 or Othello (data not shown). The addition of 0.5% PVP to the seed-soaking buffer solution circumvented the inhibitory problem because characteristic DNA fragments were consistently amplified from blight-contaminated colored seeds (Fig. 2, lanes D and E). The detection assay was shortened and simplified considerably by directly extracting the genomic DNA of bacterial cells that coated the seeds with a solution of sodium hydroxide and PVP. This method successfully yielded typical DNA products with the X4 and HB14 primers from extracts of blight-contaminated seeds (Fig. 2, lane F).

Detection of internal seed contamination. Thirty *X. campestris* pv. *phaseoli*-injected seeds were used to assess a PCR procedure for detection of internal seed infection. External contamination with *X. campestris* pv. *phaseoli* cells was detected by PCR for all individual seeds (Fig. 3, EXT). Attempts to eliminate common-blight bacterial cells that coated the seeds by brief consecutive soakings in sodium hydroxide and PBS were very effective (Fig. 3, WAS). After reextraction with sodium hydroxide, only five seeds had residual amounts of externally contaminating *X. campestris* pv. *phaseoli* DNA detected by PCR (Fig. 3, WAS, lane 2). Extracting coarsely crushed seeds with alkali was successful for detection of internal contamination with common-blight bacterial cells because the distinctive 730-bp DNA fragment for *X. campestris* pv. *phaseoli* was amplified from washed seeds (Fig. 3, INT). Although external contamination with *X. campestris* pv. *phaseoli* DNA was detected after washing, in some cases (Fig. 3, WAS, lane 2) the relative intensity of the amplified DNA bands showed clearly that the residual surface contamination of the seed contributed minimally to the population of internal pathogen DNA (Fig. 3, INT).

Concurrent detection of blight bacteria. In combination, primers HB14 and X4 were used successfully to detect contamination of batches of seeds with *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola*, either individually (Fig. 4, lanes A and B) or in mixed infections (Fig. 4, lanes C to H). Using samples of 200 seeds, three commercial seed batches were infected with *P. syringae* pv. *phaseolicola* and one with *X. campestris* pv. *phaseoli* (data not shown). These results were validated by plating the PBS-seed extracts on semiselective agar. Both bacteria were detected concurrently when the four commercial seed samples were pooled (data not shown). The sensitivity threshold of the PCR assay was assessed by adding a single *X. campestris* pv. *phaseoli*-contaminated seed and a single *P. syringae* pv. *phaseolicola*-contaminated seed to known numbers of blight-free certified seeds. Distinctive PCR fragments were detected from seed batches with 1 infected seed in 10, 100, 500, 1,000, 5,000, and 10,000 seeds (Fig. 4, lanes C to H). It was critical to perform the PCR assay immediately after the DNA extraction, particularly from large seed samples (1,000 to 10,000 seeds). The amplification efficiency was

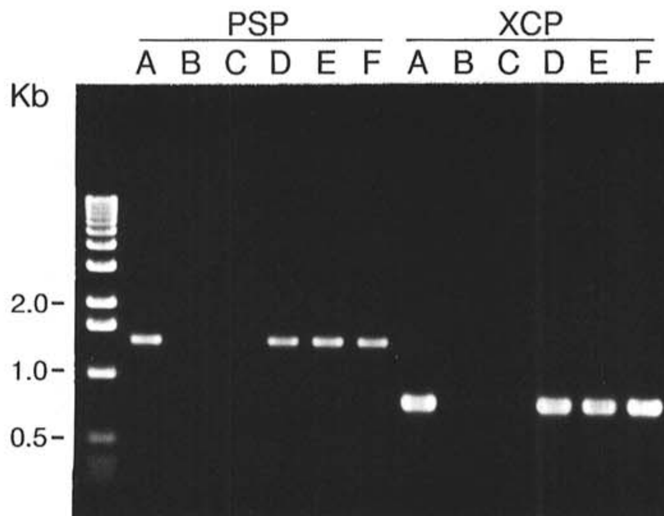

Fig. 2. Ethidium bromide-stained gel of polymerase chain reaction (PCR)-amplification products from DNA extracted from blight-contaminated bean seeds. Lanes A, white bean cultivar US1140; lanes B, D, and F, colored bean cultivar ISB82772; and lanes C and E, colored bean cultivar Othello seeds contaminated with *Pseudomonas syringae* pv. *phaseolicola* (PSP) and *Xanthomonas campestris* pv. *phaseoli* (XCP) were soaked for 16 h at 4°C in a phosphate buffered saline with (lanes D and E) or without (lanes A, B, and C) polyvinylpyrrolidone (PVP) or for 2 min in a solution of sodium hydroxide and PVP (lane F). The extracted DNA was used in PCR assays directed by HB14 (PSP) and X4 (XCP) primers. Molecular weight standard (1.0-kb ladder; BRL) was run in the left lane (unmarked); numbers on the left indicate sizes in kilobases.

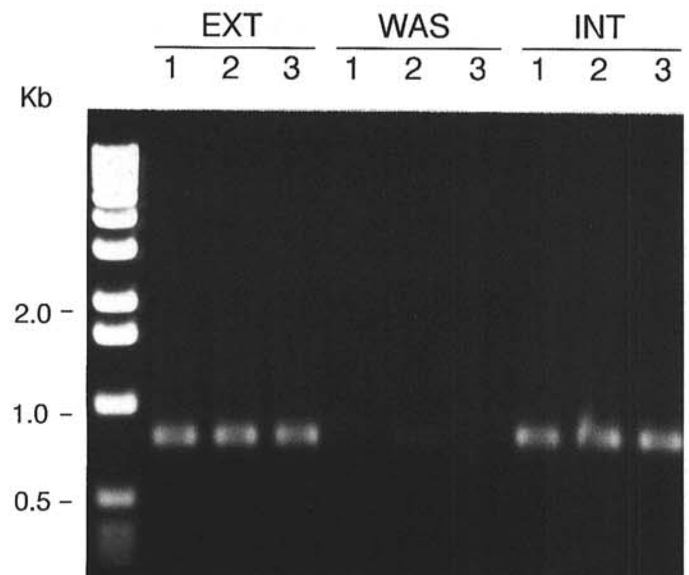

Fig. 3. Ethidium bromide-stained gel of polymerase chain reaction-amplification products from DNA extracted from common blight-contaminated bean seeds. Lanes 1, 2, and 3, three individual colored bean cultivar ISB82772 seeds contaminated with *Xanthomonas campestris* pv. *phaseoli* were soaked for 2 min in a solution of sodium hydroxide and polyvinylpyrrolidone to extract DNA from the common-blight bacteria that coated the seeds (EXT). The seeds were washed twice in phosphate buffered saline and were reextracted with the alkaline solution (WAS). The seeds were crushed and treated again with alkali to extract DNA from internally contaminated bacteria (INT). Molecular weight standard (1.0-kb ladder; BRL) was run in the left lane (unmarked); numbers on the left indicate sizes in kilobases.

significantly reduced when DNA samples were stored for even a few hours at 4 or –20°C.

DISCUSSION

A quick and sensitive procedure was developed for specific and concurrent detection of *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* in bean seeds. Discrete DNA fragments related to common and halo blight bacteria were specifically amplified from target DNA templates extracted by alkali from contaminated bean seeds. This method allowed for rapid detection of either individual or mixed seedborne contamination of common and halo blight agents in samples of 10,000 seeds.

Selection of DNA fragments unique to *X. campestris* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* was imperative in the development of a specific nucleic acid-based detection system. High melting temperature primers, designed from a specific repetitive DNA segment of *X. campestris* pv. *phaseoli* (7), have been employed in a stringent two-temperature PCR assay to detect common-blight bacteria in bean leaf lesions (1). Priming at high temperature prevented nonspecific amplification and shortened the PCR assay considerably (1).

In this study, X4 primers were chosen for detection of common-blight bacteria because of their specificity for *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* (1). As expected, these primers were specific in directing the amplification of a 730-bp fragment only from genomic DNA of the nine *X. campestris* pv. *phaseoli* strains tested. The same criteria, high G+C content and specificity, also were used to design primers for *P. syringae* pv. *phaseolicola*. In previous reports, segments of the phaseolotoxin gene cluster ("Tox") of *P. syringae* pv. *phaseolicola* were used in dot-blot hybridization and amplification assays for halo blight pathogens (15,21,26). Phaseolotoxin production is a characteristic trait of this pathogen (13), although *P. syringae* pv. *glycinae* strains produce closely related compounds (9). In this study, G+C-rich HB14 primers were designed from the sequence of a 1.9-kb DNA fragment of the Tox gene cluster amplified from *P. syringae* pv. *phaseolicola* genomic DNA with the HM6 and HM13 primers devised by Prosen et al. (15). HB14 primers generated characteristic PCR products only from the 19 *P. syringae* pv. *phaseolicola* strains tested. No DNA fragments were amplified from the 62 other bacteria, which included two strains of *P. syringae* pv. *glycinea* that may produce phaseolotoxin-like compounds (9).

Pathogenic Tox negative ("Tox–") strains of *P. syringae* pv. *phaseolicola* have been reported (13), although they appear to be of little epidemiological importance in halo blight of bean (21). Many of the pathogenic haloless Tox– strains still possess the phaseolotoxin gene cluster in their chromosome and have been detected by PCR assay with primers devised from the Tox gene cluster (21). In this study, *P. syringae* pv. *phaseolicola* strains were not evaluated for phaseolotoxin production, but a few strains produced haloless lesions on susceptible bean cultivars. Amplification of the 1.4-kb DNA fragment with the HB14 primers was similar for the haloless and the halo-producing strains. The HB14 primers were designed from the same DNA segment targeted by Schaad et al. (21) in a PCR assay on Tox– strains, and thus, they are likely to detect this subgroup of *P. syringae* pv. *phaseolicola* strains.

DNA extracts from colored seeds were refractory to PCR amplification, whereas this was never observed with white seeds. Prolonged soaking of seeds has a negative effect on PCR amplification (16). It is probable that inhibitory factors are released from colored seeds into the extraction buffer. Similar inhibition of PCR amplification was observed with extracts from relatively old common-blight lesions on bean leaves (P. Audy, *unpublished*). The addition of PVP to the extraction buffer restored DNA amplification. PVP improves the stability of enzymes by inactivating strongly reactive phenolic impurities that likely interfere with the DNA polymerase activity. PVP was only effective when added in the extraction buffer; addition at subsequent steps did not restore the DNA amplification activity. Moreover, its beneficial action was transient, because PCR amplification was reduced in PVP-treated samples kept for a few hours at room temperature, as well as at 4 and –20°C.

The alkaline DNA extraction procedure presents significant advantages over conventional methods of preparing DNA because organic solvents and DNA precipitation are not required. In practice, the preparation of suitable DNA extracts for PCR from externally infected seeds required less than 2 min per sample for batches of 100 seeds or less. Moreover, the method is not destructive; bean seeds briefly wetted with sodium hydroxide and washed with water had a germination performance similar to that of untreated seeds (P. Audy, *unpublished*). This feature could be valuable in breeding programs in which selection for blight resistance in early generations is desirable.

A major issue in PCR-based diagnosis involves the potential for cross-contamination while processing samples. Because of the exceptional sensitivity of PCR, reuse of sampling containers, despite great caution in managing materials and operations, may result in contamination of samples. The cost of purchasing and storing several containers, the time and labor required for acid-depurination and washing treatments between samples, and the facilities needed to achieve the necessary decontamination may discourage potential users from choosing PCR technology for large-scale diagnosis. This problem was overcome by the use of disposable polypropylene bags in which samples were collected and processed through the extraction procedures. The usual protocol for bean blight testing consists of individually extracting three groups of 10,000 seeds from a given lot (23,27). This could be handled easily with a high-capacity centrifuge with buckets that take six polypropylene bags containing 2,000 seeds each. By using a brief centrifugation (2 min) to recover the lysis extract, the volume of the alkaline extraction mixture required to wet the seeds was reduced to 15 μl per seed for large batches of seeds (>500). Extracting bacterial DNA in a minimal volume further increased the sensitivity of our PCR assay. It was unnecessary to run more than a single PCR amplification even at very low infection levels.

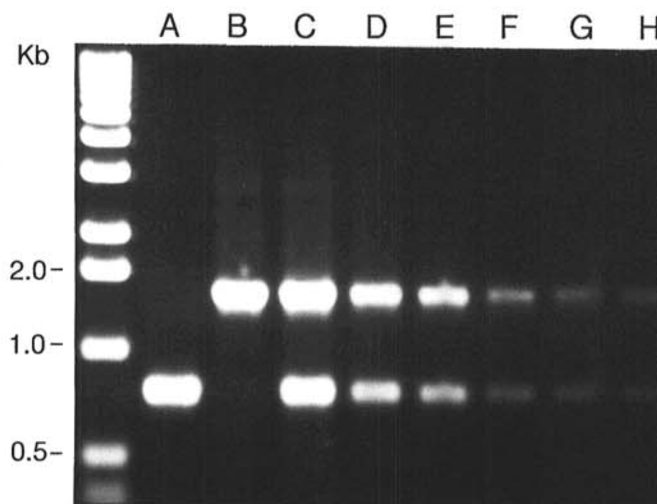

Fig. 4. Ethidium bromide-stained gel of polymerase chain reaction-amplification products to assess the sensitivity of the assay for concurrent detection of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola* in bean seeds. HB14 and X4 primers were used simultaneously to direct amplification of DNA fragments from extracts of lane A, 1 *X. campestris* pv. *phaseoli*- or lane B, 1 *P. syringae* pv. *phaseolicola*-contaminated seed in 10 blight-free certified seeds or from 1 mixed-contaminated seed in 10, 100, 500, 1,000, 5,000, and 10,000 blight-free certified seeds (lanes C to H, respectively). Molecular weight standard (1.0-kb ladder; BRL) was run in the left lane (unmarked); numbers on the left indicate sizes in kilobases.

It is important to note that, as in most serological approaches, this PCR assay does not discriminate between live and dead pathogen cells. However, this aspect is likely to be of minor importance for diagnosis of seasonally harvested seed material, because common-blight bacteria can survive in bean seed for more than 30 years (17). Therefore, an incorrect diagnosis resulting from the absence of live pathogenic cells in a blight-contaminated seed batch is implausible. On the other hand, dead cells may provide a significant amount of target DNA and have a beneficial effect on the sensitivity of a detection method. This PCR assay has great potential for commercial-scale diagnosis.

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