A Combined Biological and Enzymatic Amplification (BIO-PCR) Technique to Detect *Pseudomonas syringae* pv. *phaseolicola* in Bean Seed Extracts

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Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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ABSTRACT

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A polymerase chain reaction (PCR) method, hereafter referred to as BIO-PCR, that combines biological and enzymatic amplification of PCR targets and simplified procedures for sample processing is described for the detection of *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. Seeds are soaked overnight following standard protocols and aliquots of the extracts are plated onto a general agar medium. After 45–48 hr of incubation, the plates are washed with water to remove bacterial cells and aliquots of a pooled washing are subjected to two consecutive rounds of PCR, without prior DNA extraction, using "nested" pairs of primers designed to amplify a segment of the organism's *tox* (phaseolotoxin) gene region. Positive detection was reproducibly obtained at near-limit concentrations of the pathogen in the seed wash. Advantages of BIO-PCR over existing PCR techniques include the elimination of false positives resulting from the presence of dead cells that may be present in the seed, elimination of false negatives due to potential PCR inhibitors in seed extracts, increased sensitivity of detection, and no need for DNA extraction prior to amplification. Accordingly, BIO-PCR should prove useful for routine detection of other bacterial pathogens of quarantine importance. The primers detected some nontoxigenic strains of the pathogen, which evidently contain part of or the entire *tox* gene cluster.

Additional keywords: bacteria, halo blight of beans.

Pseudomonas syringae pv. *phaseolicola* is a serious seedborne bacterial pathogen of beans worldwide, causing the halo blight disease (31,37). Because of the rapid spread of the pathogen under suitable weather conditions, even low levels of primary infection can result in severe epidemics (36). For example, seed infestation levels of 0.02% (37) or 0.05% (33) resulted in severe halo blight in Wisconsin and France, respectively. A zero tolerance level of the pathogen in stock seed is enforced by seed-producing states such as Idaho (37). All seed fields are inspected and, if halo blight is found, the field is destroyed (37). Available seed assays include testing liquid suspensions or seed-soak extracts of commercial seeds by *1)* immunofluorescence combined with isolation on a general plating medium, followed by pathogenicity tests (35), *2)* isolation on a semiselective agar medium (modified sucrose peptone, MSP) (18) followed by pathogenicity tests (18), *3)* isolation on MSP medium followed by microbiological assay for phaseolotoxin (12), or *4)* testing seed-soak extracts of cull, field-thrashed seeds by plant injection (37).

Although the toxin assay (12) is less time-consuming than pathogenicity tests, its application is limited by the need to identify suspect colonies on MSP medium, which is problematic in samples carrying large numbers of saprophytic or other bean pathogenic bacteria. This is also a major disadvantage of the other methods mentioned above. In addition, the toxin assay would not detect any Tox− strains, which are still pathogenic (20).

A polymerase chain reaction (PCR) (27) technique that amplifies a segment of the genomic phaseolotoxin gene cluster (20) by using a single pair of primers was recently developed (22). The method is specific for *P. s. phaseolicola,* but requires time-consuming and cost-adding Southern blots to detect the small numbers of pathogen cfu found in some seed extracts. In addition, seeds of some plants evidently contain inhibitors of PCR (22, 23,32), as is the case with soil (2), food products (25), and plant leaves (5,26), which are not easily removed by standard extraction procedures. For example, in our previous studies (22) DNA from seed extracts containing relatively small numbers of pathogen cfu (on average 12 cfu per reaction) gave variable results in replicate amplifications. In contrast, preliminary tests with direct nested PCR (see Materials and Methods) carried out with pure culture aliquots containing 2–3 cfu of *P. s. phaseolicola* per reaction consistently gave strong PCR bands. Seed lots with relatively low levels of saprophytic bacteria can be assayed by agar plating (18) and suspect colonies of *P. s. phaseolicola* easily removed and tested by PCR or other methods. However, in many seed lots the extracts must be diluted 10^{-2} in order to reduce the numbers of saprophytes so that colonies of *P. s. phaseolicola* can be easily identified. Another general problem with PCR techniques is their inability to differentiate between dead cells and live cells, which is important in many phytosanitary applications.

To overcome these problems we have introduced an agar plating step prior to PCR analysis. This modification, which we have

named BIO-PCR, provides the added benefit of "biological amplification" of the PCR targets in the seed extract. The technique greatly improves detection of the pathogen in seed washings with low levels of contamination and greatly reduces the chances of detecting dead cells or free DNA. Aliquots of a seed extract are first plated onto King et al's medium B (KB) and after 45–48 hr pooled washings from the plates are used for direct nested PCR. A preliminary report has been published (29).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas s. phaseolicola* strain C-199 originated from bean plants in Idaho (N. W. Schaad) and was used in previous studies on DNA probes (28) and PCR (22). *Pseudomonas s. phaseolicola* strains NPS3121 and 4419 have been described previously (20). The phenotypically Tox− *P. s. phaseolicola* strains were obtained from K. Rudolph (Institute fur Pflanzenpathologie und Pflanzenschutz, George August Universitat, Gottingen, Germany): GSPB592 and GSPB593, single colony isolates from strain 0458 (M. L. Moffett, Department of Primary Industries, Brisbane, Australia), which was originally isolated from *Glycine weightii;* GSPB792, reisolated from bean after inoculation with strain W51-32, a Tox− strain originally isolated in the Netherlands by J. C. Walker, Department of Plant Pathology, University of Wisconsin, Madison; Strains GSPB606, GSPB607, and GSPB612, isolated by K. Rudolph from plants in field plots that had been inoculated with strain GSPB792. All cultures were maintained on KB (15) agar slants and grown in Luria-Bertani (LB) (16) liquid medium. Permanent stocks were stored at −80 C (19). Unless otherwise stated, *Pseudomonas* strains were grown at 28 C.

Oligonucleotide primers. The following primers were selected from a 2.6-kb segment of the *tox* region (21,22,28) that has been sequenced (E. Hatziloukas and N. J. Panopoulos, *unpublished*), by using the primer analysis program OLIGO (National Biosciences, Hamel, MN): P 5.1 : 5′AGCTTCTCCTCAAAACACC-TGC3′; P 3.1:5′TGTTCGCCAGAGGCAGTCATG3′; P 5.2 : 5′TCGAACATCAATCTGCCAGCCA3′; P 3.2 : 5′GGCTTTT-ATTATTGCCGTGGGC3′. The binding sites for primers P 5.1 and P 3.1 are located outside those of primers P 5.2 and P 3.2 in the above segment (Fig. 1); therefore, the first two primers are referred to as the external primer pair and the later two as the internal or nested primer pair.

Comparison of different DNA extraction methods to "direct PCR." To determine if a more simplified method could be used for sample processing the following methods were tested: *1)* the hexadecyltrimethylammonium bromide (CTAB) method (1) modified by omitting the second phenol/chloroform/isoamyl alcohol deproteinization step and adding 5 μg of yeast tRNA (Sigma Chemical Co., St. Louis, MO) per sample to facilitate nucleic acid precipitation (22); *2)* aliquots of cell suspensions were boiled for 10 min and placed immediately on ice before amplification; *3)* cell suspensions were boiled for 10 min, tRNA and isopropanol were added, and further treated as in the CTAB method above; *4)* direct amplification of small aliquots of bacterial cultures was performed without prior DNA extraction or other sample processing ("direct PCR"). Identical aliquots from the same liquid culture of *P. s. phaseolicola* C-199 (adjusted to contain between 4 and 10 cfu) were used in the above four procedures. Samples were amplified by nested PCR and the products analyzed by electrophoresis, as described below. Six replicates, each a separate DNA or bacterial preparation, were tested in the first experiment and four in the second experiment.

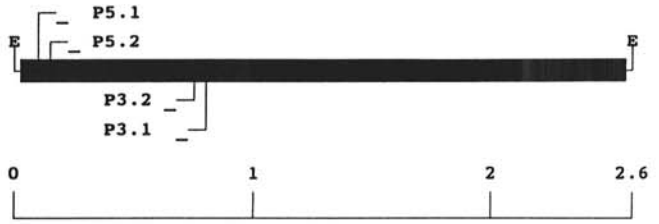

Fig. 1. Position of the PCR primers in the 2.6-kb *Eco*RI *tox* cluster segment of *Pseudomonas syringae* pv. *phaseolicola.*

DNA probes and hybridization. Two different probes were used in the Southern hybridization analysis. First, for the determination of sensitivity of the method and for the detection of the pathogen in water extracts of bean seed, the probe, a 0.45-kb DNA fragment of the 2.6-kb *Eco*RI segment, was synthesized and labeled by PCR as follows: 40 ng of *P. s. phaseolicola* C-199 genomic DNA, was amplified by using the external primer pair, as described below. A 2-μl aliquot of a 1:10 dilution of the product of the first amplification round was re-amplified under identical reaction and cycling conditions but using the internal primer pair and a deoxynucleotide triphosphate mixture containing digoxigenin-11-dUTP, as the labeling nucleotide (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The resulting DNA was used as hybridization probe following the protocol recommended by the supplier of the labeling nucleotide. Second, for the hybridization analysis of the genomic DNAs from the *P. s. phaseolicola* Tox− strains, as well as their nested PCR products (see below), we used as probe the 2.6-kb *Eco*RI fragment (Fig. 1), derived from the *tox* cluster of *P. s. phaseolicola* NPS3121, described earlier (20–22,28). This fragment was labeled by using the nonradioactive DNA labeling kit (Boehringer-Mannheim) according to the supplier's instructions.

Capillary transfer and cross-linking of DNA to Nytran membranes (Boehringer-Mannheim) were done following standard procedures (1,16). Prehybridizations and hybridizations were carried out according to the instructions of the membrane supplier (Boehringer-Mannheim) and labeled nucleic acids were detected by using the Genius immunoblot kit (Boehringer-Mannheim).

PCR protocols. Standard PCR reactions were carried out by using the external primer pair. Reactions were routinely done in quadruplicate with the following profile: an initial 2 min incubation at 94 C, a manual "hot start" step (3) at 80 C, 25–30 cycles (1 min at 94 C, 1 min at 58 C, and 2 min at 72 C), and a final extension step of 8 min at 72 C. Double-nested (hereafter referred to simply as nested) PCR reactions consisted of standard PCR with the external primer pair, followed by re-amplification of 2 μl of 10-fold diluted products with the internal primer pair for 25 cycles. All amplifications were carried out in 0.5-ml thin-wall tubes, in a final volume of 50 μl, and under a layer of mineral oil (Perkin Elmer-Cetus, Norwalk, CT) in a Perkin Elmer-Cetus Model 480 DNA Thermocycler. Reaction mixtures contained the following ingredients at the given final concentrations: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 80 μM each of dATP, dCTP, dGTP, and dTTP; 0.2 units of *Taq* DNA polymerase (Perkin Elmer-Cetus); and, 0.5 μM of each primer. Stock solutions were stored in aliquots at −20 C and never used more than five times. Sterile double distilled water (SDDW) was used for all solutions and dilutions above. To help avoid carry-over contamination, duplicate samples and SDDW controls were routinely included in each experiment and PCR conducted in a separate laboratory. All experiments were repeated at least once. Amplification products were analyzed by electrophoresis on agarose gels that were either stained with ethidium bromide (EtBr) as described (16) or blotted onto Nytran membranes for hybridization.

Sensitivity of detection. The sensitivity threshold for the external and internal primer pairs in the standard and nested PCR format was determined with the following procedure. Triplicate 0.1-ml samples of 10^{-6}, 10^{-7}, 0.5×10^{-7}, and 10^{-8} serial dilutions of a liquid culture of *P. s. phaseolicola* NPS 3121 (initial $OD_{600} = 0.1$) were plated onto KB plates to determine cfu per milliliter. Duplicate 1.0-ml aliquots from each dilution were stored at −20 C. Genomic DNA was extracted from these stored aliquots by the CTAB method modified as above and adding 5 μg of yeast tRNA per sample to facilitate nucleic acid precipitation; the final nucleic acid pellet was rinsed with 70% ethanol (−20 C), dried, and re-suspended in 30 μl of TE buffer. PCR analysis was carried out in 10 μl aliquots of these extracts. DNA was

extracted from a liquid culture (OD_{600} = 0.1) by the modified CTAB method (without tRNA addition) and its concentration determined spectrophotometrically (16). Serial dilutions of this extract were made in TE buffer and 10 μl aliquots used for PCR analysis. PCR products were analyzed as described above.

Detection of *P. s. phaseolicola* in bean seed extracts. Commercial bean seeds (cv. Great Northern White) were obtained from seed companies in California and from a grocery store in Frederick, Maryland. A pool of 2 kg of these seed stocks that had approximately 1×10^4 cfu of saprophytic bacteria per milliliter of seed wash was selected for quantification of detection thresholds. The mixture was negative for *P. s. phaseolicola,* based on agar plating (18), direct PCR, and BIO-PCR assays (as described below).

Seed-water extracts were prepared by soaking 225 g (~1,000) seeds overnight in 500 ml of 0.01% Tween 20 in sterile water, as described by Mohan and Schaad (18). Aliquots (0.1 ml) of the seed extracts were plated in triplicate onto KB- and MSP-agar plates to determine bacterial populations and confirm the absence of *P. s. phaseolicola*. A second set of aliquots (1 ml) was stored at −20 C, and analyzed later by direct PCR (without DNA extraction or other treatments), by using the standard or nested format described above. A third set of aliquots was used to prepare seed extract stocks containing the following expected numbers of *P. s. phaseolicola* cfu per 0.1 ml: 10–20, 1–2, and <1. These stocks were made by mixing 0.5 ml of serially diluted aliquots of an overnight culture adjusted to 0.1 OD_{600} with 4.5 ml of undiluted or 10^{-1} diluted seed extract. Similarly, 0.5 ml of each of the above dilutions of the bacterial culture was mixed with 4.5 ml of SDDW (water control), to determine actual *P. s. phaseolicola* cfu added. A portion of the original seed extract was set aside prior to adding *P. s. phaseolicola* to serve as a seed extract control. Each of the seed extract mixtures, water controls, and seed extract controls was plated onto KB-agar plates (0.1 ml per plate). Extract treatments with expected *P. s. phaseolicola* cfu of 10–20, 1–2, and <1 per 0.1 ml consisted of two, eight, and eight plates, respectively. Water control treatments with the same pathogen cfu consisted of two, four, and eight plates, respectively. The seed extract controls were plated on two (undiluted) and four (10^{-1} dilution) plates, respectively. One-half the number of plates from each of the above treatments were incubated for 4 days to determine the actual cfu of saprophytic bacteria and *P. s. phaseolicola*. The remaining plates from each mixture or control were incubated for 45 hr (*P. s. phaseolicola* colonies ~0.5–1.0 mm in diameter), washed individually three times, each time with 1 ml of SDDW. The combined washes from each plate (total volume 3 ml) were used directly for PCR analysis (35 μl per reaction), as described above, or stored at −20 C. This experiment was repeated twice.

Detection of Tox− strains. None of the six Tox− strains caused formation of chlorotic halos, although they all produced water-soaked lesions on the bean cultivar Red Kidney (K. Rudolph, *personal communication*). These strains were examined for the presence of *tox* sequences in their chromosome. *Pseudomonas* strains and *Escherichia coli* were grown at 18–20 C and 37 C, respectively, for the phaseolotoxin assay. Production of phaseolotoxin was determined by the standard microbiological assay (30) using *E. coli* HB101 as the indicator and strains NPS3121 and 4419 of *P. s. phaseolicola* as positive controls.

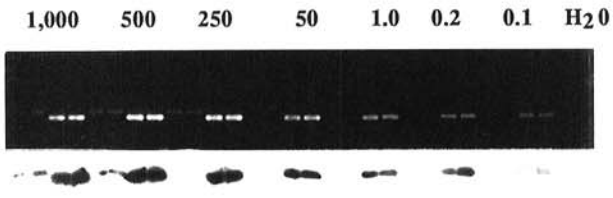

Fig. 2. Detection threshold of *Pseudomonas syringae* pv. *phaseolicola* DNA by standard and nested PCR. A group of four lanes is shown for each DNA concentration. Two left lanes in each group correspond to duplicate PCR reactions with external primers; two right lanes, to duplicate reactions with nested primers. Rightmost lane shows sterile distilled water control. Upper panel shows ethidium bromide–stained bands (white on black background); lower panel, bands detected by Southern hybridization (black bands on white background). Size of standard and nested PCR products is 0.5 and 0.45 kb, respectively. Numbers on top indicate picogram of DNA used per amplification reaction.

RESULTS

Sensitivity of detection. Both the standard and nested PCR formats yielded single discrete product bands (0.5 kb with the external primers and 0.45 kb with the internal primers) (Fig. 2). The detection threshold for serially diluted cells was 1,200 cells for the standard PCR format with external primers and 1–2 cells for the nested PCR format, based on Southern hybridization. For purified genomic DNA, the detection threshold was 250 pg with external primers, and 0.1 pg (2,500-fold lower) with the nested primers, based on EtBr staining and Southern hybridization (Fig. 2). In a separate experiment, eight of eight and four of six samples containing 1.0 and 0.1 pg, respectively, were positive with nested primers. The most dilute sample in that experiment contained 0.02 pg and two of four samples were weakly positive (weak bands) by nested PCR. Assuming a genome size for *P. s. phaseolicola* equal to that of *Pseudomonas aeruginosa* (i.e., 5.85 Mb) (11,22), 250 and 0.02–0.1 pg DNA correspond to ~4×10^4 and 3–15 genome equivalents per reaction (one equivalent corresponding to one bacterial cell), respectively. Our calculation was based on Avogadro's standard number, where 1.0 mol of *P. s. phaseolicola* genomes contain 6.023×10^{23} molecules, and one genome equivalent is approximately 6.3 fg.

Comparing methods of DNA extraction to "direct PCR." Direct PCR (see Materials and Methods) gave the most consistent and reproducible results. In one experiment, out of six samples tested, 6, 2, 0, and 3 were scored positive by direct PCR, modified CTAB, boiling only, and boiling plus precipitation, respectively. In a second experiment, out of four samples tested, 3, 4, 0, and 0 were scored positive by direct PCR, modified CTAB, boiling, and boiling followed by precipitation, respectively.

Detection of *P. s. phaseolicola* added to bean seed extracts by BIO-PCR. In preliminary experiments diluted aliquots of pure culture of *P. s. phaseolicola* containing 10^3 cfu per milliliter were normally positive when tested by direct nested PCR whereas seed extracts containing the same number of added cells were often negative. In contrast, similar extracts with as few as 10 added cfu of *P. s. phaseolicola* per milliliter were positive when tested by BIO-PCR (see Materials and Methods). In a representative BIO-PCR experiment, six of eight PCR samples from washings of separate agar plates containing approximately 110 saprophytic colonies and 1 or 2 colonies of *P. s. phaseolicola* (based on the water control "sister" plates), were positive by nested PCR (Table 1). No *P. s. phaseolicola* were observed on KB- or MSP-agar plates of assays of aliquots of the seed extracts that had been set aside prior to adding *P. s. phaseolicola*. Furthermore, BIO-PCR assays from the undiluted and 10^{-1} diluted control seed extracts, as well as independent assays of such extracts stored at −20 C by direct nested PCR, were negative.

Detection of Tox− strains. The use of the *tox* gene cluster, either as a DNA probe (28) or as a PCR target (22, and this study), or the phaseolotoxin assay itself (12) to detect *P. s. phaseolicola* in bean seed has the possible disadvantage that "haloless" (toxin nonproducing strains) (13,17) could go undetected. Although Tox− strains in general apparently have little or no epidemiological importance in halo blight of beans (N. W. Schaad, *unpublished data*) they are still pathogenic (13,17,20). Naturally occurring Tox− strains have not been genetically characterized. It is possible that such strains contain a silent *tox* cluster or portions thereof and, thus, can potentially revert to a Tox+ phenotype in nature. Accordingly, six cultures received as Tox− strains (GSPB592, GSPB593, GSPB606, GSPB607, GSPB612, and GSPB792) were examined in this study. The latter four strains gave the expected 0.5 and 0.45 kb bands after standard and nested PCR, respectively, and their total DNA extracts contained the 2.6-kb *Eco*RI *tox* gene fragment, based

on Southern hybridizations carried out by using this fragment as a probe (22) (Table 2). Strains GSPB592 and GSPB593 did not give any PCR bands and did not contain any homology to the above probe. Strain GSPB792, although assumed to be Tox− (based on its inability to form chlorotic halos on bean), produced phaseolotoxin, as determined by the *E. coli* microbiological assay. The other five GSPB strains did not produce any detectable toxin.

DISCUSSION

The procedure developed in this study combines several technical improvements to our previous PCR method for the detection of *P. s. phaseolicola* in bean seed (22). Specifically, a plating step on an ordinary (nonselective) agar medium is followed by direct amplification of plate washes, without prior DNA extraction, and a two-stage PCR using different pairs of primers, one nested within the other. The technique, called BIO-PCR, has many advantages over standard PCR methods for routine detection of seedborne pathogens; among them are increased sensitivity, simplicity, and detection of only live cells. Accordingly, it should facilitate the implementation of PCR-based screening of commercial bean seed lots for *P. s. phaseolicola* contamination, especially in laboratories that are not fully equipped, or lack personnel trained in various techniques connected with PCR applications. Additionally, it should be very useful for studying epidemiology, host-pathogen relations, biopesticide risk assessment, and environmental sampling including soil and water. Unlike our standard PCR method (22), our BIO-PCR technique has not yet been tested with naturally contaminated bean seed lots. However, a comparative test has been organized in cooperation with R. L. Forster, University of Idaho, and S. K. Marquardt, North Dakota State Seed Lab, to compare the reliability of BIO-PCR with the standard methods used by the Idaho State Department of Agriculture and North Dakota State Seed Lab, respectively, for detection of bean seeds contaminated with *P. s. phaseolicola*.

The agar-plating step accomplishes several different objectives: it reduces, and perhaps eliminates, the detection of dead propagules and cell-free DNA, effectively removes PCR inhibitors that may be present in the original seed wash sample, and permits the "biological amplification" of PCR targets prior to their enzymatic amplification. Agar plating is similar in principle to liquid enrichment techniques (e.g., 8), but probably removes PCR inhibitors more effectively by diffusion into the agar matrix. It should be noted that the KB medium used in our study is not selective for the halo blight pathogen. The semiselective MSP medium provides such an option for *P. s. phaseolicola,* but it is known to reduce the growth of some strains (18). We used KB because it is the standard general plating medium for pseudomonads. Other formulations of media may increase the recovery of *P. s. phaseolicola* from seed washes and thereby increase the sensitivity of the method even more. The use of cells washed from agar media for amplification, coupled with the nested PCR format, greatly increases the sensitivity of detection in seed lots with low levels of *P. s. phaseolicola* contamination by allowing use of a much greater volume of sample (100 μl for agar plating versus 10 μl for standard PCR), and eliminates costly Southern hybridization. We did not test samples of 400–500 μl for plating but such volumes could be used. Another advantage of BIO-PCR is that quantitative data on viable pathogen population can be obtained by dilution-endpoint analysis if samples are not heavily contaminated with saprophytes. Although quantitation of *P. s. phaseolicola* contamination in bean seed is currently not necessary because a "zero tolerance" is followed (37), quantification of pathogen propagules would be desirable in other cases. With BIO-PCR, use of hazardous chemicals such as phenol is avoided, cells are not lost during DNA extraction procedures, and the method is considerably less technical due to the elimination of the DNA extraction and Southern hybridization steps. In addition, samples of bean seeds do not need to be mailed to a laboratory for assay. The seed extracts can be prepared and plated onto KB agar at the site of origin and then either mailed immediately overnight to a PCR testing laboratory or incubated for 43 hr and the pooled plate washings collected and mailed frozen. Immediate mailing of the agar plates would compensate for the 43 hr required for incubation on KB plates.

The detection threshold that can be achieved by BIO-PCR at its present stage of development is 3×10^3 cfu per sample of 3,000 seeds if the seeds are washed in 1,500 ml of buffer, as recommended (18) and five samples of 100 μl are plated onto

TABLE 1. Detection of *Pseudomonas syringae* pv. *phaseolicola* (PSP) added to bean seed extract by BIO-PCR[a]

Expected no. of cfu PSP per plate	Treatment	Plate[b] group	Observed no. cfu per plate: PSP	Observed no. cfu per plate: Other bacteria	BIO-PCR presence of 0.45 kb band
10–20	Undiluted seed extract	I	0[c]	C[d]	NA[e]
		II	NA	NA	+[f]
	Water control	I	12	NA	NA
		II	NA	NA	+
1–2	10^{-1} dilution of seed extract	I	2,2,2,2	97,108,118,122	NA
		II	NA	NA	+,+,+,−
	Water control	I	0,2	NA	NA
		II	NA	NA	+,−
<1	10^{-1} dilution of seed extract	I	0,0,0,0	85,105,106,115	NA
		II	NA	NA	+,+,+,−
	Water control	I	1,1,0,0	NA	NA
		II	NA	NA	+,+,−,−
0[g]	Undiluted seed extract	I	0	C	NA
		II	NA	NA	−
	10^{-1} dilution of seed extract	I	0,0	88,124	NA
		II	NA	NA	−,−

[a] Four and one-half ml of seed extract or sterile double distilled water (used as control) mixed with 0.5 ml of different diluted suspensions of *P. s. phaseolicola* and 0.1 ml aliquots from each mixture plated onto replicated KB agar plates. Treatments designed to result in different expected numbers of *P. s. phaseolicola* cfu per plate and included: seed extract–*P. s. phaseolicola* mixtures with 10–20, 1–2, and <1 *P. s. phaseolicola* cfu per plate (2, 8, and 8 plates, respectively); water controls with same pathogen cfu per plate as in above treatments (2, 4, and 8 plates, respectively); and seed extract (negative control) taken before adding *P. s. phaseolicola* (2 and 4 plates for undiluted and 10^1-diluted extract, respectively). After 45 hr, half the plates (group II plates) for each treatment were washed three times with 1 ml of sterile distilled water; pooled washings from each plate (3 ml) were used for BIO-PCR analysis, as described in text; the other half (group I plates) were incubated for 96 hr to determine actual *P. s. phaseolicola* cfu in the water controls or total bacteria in the other plates for each treatment.

[b] Group I represents plates used to determine cfu and group II those used for BIO-PCR.

[c] Colonies of *P. s. phaseolicola* could not be visually identified among those of the other bacteria (this does not necessarily mean that such colonies were not present).

[d] Confluent.

[e] Not applicable.

[f] Presence (+) or absence (−) of band for each replicate.

[g] Negative control; sample taken before adding *P. s. phaseolicola* to the bean seed extract.

TABLE 2. Analysis of Tox− strains of *Pseudomonas syringae* pv. *phaseolicola* by nested PCR and Southern hybridization

Strain	Toxin assay[a]	0.45-kb PCR band[b]	Southern hybridization: PCR products[c]	Southern hybridization: Genomic DNA[c]
NPS 3121	+	+	+	+
GSPB792	+	+	+	+
GSBP606	−	+	+	+
GSPB607	−	+	+	+
GSPB612	−	+	+	+
GSBP592	−	−	−	−
GSPB593	−	−	−	−

[a] Presence (+) or absence (−) of inhibition zone in *Escherichia coli* phaseolotoxin bioassay (30).

[b] Presence (+) or absence (−) of ethidium bromide–stained band after nested PCR.

[c] Presence (+) or absence (−) of hybridizing band.

KB. This means that contaminated seeds must contain greater than 3×10^3 cfu because the extraction efficiency is not 100%. Extracts of seed batches containing pathogen population levels below that level would most likely not be detected.

To our knowledge this is the first report of the successful detection of a pathogen in the range of 2–3 cells per milliliter of original sample by direct PCR (i.e., without extraction of DNA). Such high sensitivity would not be possible without BIO-PCR due to dilution factors alone.

Various forms of nested PCR have been employed in diagnostic and other applications (e.g., 4,6,7,9,14,24,34). These formats generally improve the sensitivity of PCR-based detection. In our previous study (22) a standard PCR format was employed and Southern hybridization was necessary to detect PCR products at low initial target cell/DNA concentrations in bean seed screening. This is most likely due to the fact that the *tox* gene is present as a single copy. If multiple target sequences were present, nested primers may not be needed. However, the uniqueness of the *tox* gene to *P. s. phaseolicola* far outweighs any disadvantage in low copy numbers. The present procedure gives sufficient final PCR product yield for detection of *P. s. phaseolicola* or its DNA at near-limit initial target concentrations by simple EtBr staining.

It is known that Tox− strains retain their pathogenic capacity (13,17,20), although in general, they seem to be of little or no epidemiological importance in halo blight of beans (N. W. Schaad, *unpublished data*). Naturally occurring Tox− strains have not been genetically characterized and it is possible that such strains can revert to Tox+, as may be the case with strain GSPB792, which originated from the haloless (Tox−) W51-32, but in our hands, in culture, proved to be Tox+. If reversion to Tox+ does occur, the detection of nontoxigenic strains might be as important as the detection of toxigenic ones. Such strains could spontaneously become fully virulent and generate epidemics. To this end we investigated whether Tox− strains still possess *tox* sequences. The results presented in Table 2 demonstrate that at least some of the nontoxigenic strains indeed contain such sequences. Further examination (data not shown), revealed that these strains contain an extended (and possibly the entire) region of the *tox-argK* gene cluster (10,20; E. Hatziloukas et al, *unpublished*). To our knowledge this is the first report that Tox− strains maintain this gene cluster in their chromosome, and thus the ability to detect this subgroup is a useful feature of our method.

On the basis of the present study, the following protocol is suggested: Soak three samples of 1 kg of seed (approximately 3,000 seeds) overnight in 1.5 L sterile water or buffer at 4 C, as previously described (18). Plate 100-μl aliquots of the seed extract onto each of five KB- and MSP-agar plates. After 45–48 hr at 25–28 C, wash each KB plate three times with 1 ml of sterile distilled water and pool into one sample. Keep the MSP plates 4 days for visual (tentative) identification of *P. s. phaseolicola* colonies, as described (18). Store the plate washes at −20 C or use immediately for PCR analysis. Mix 35 μl of plate wash with PCR buffer, external primer pair, and deoxynucleoside triphosphates (0.5 μM and 80 μM final concentration, respectively), add SDDW to a final volume of 50 μl and cover with two drops of mineral oil. Microfuge samples, place in thermocycler set at 80 C, add *Taq* polymerase (1 unit per reaction), incubate for 2 min at 94 C, and initiate 25–30 cycles of 1 min at 94 C, 1 min at 58 C, and 2 min at 72 C, with a final extension step of 8 min at 72 C. Remove samples and place on ice. Dilute 2 μl of amplification products with 18 μl of SDDW and use 2 μl for a second round of PCR as above, but with the internal primer pair. Detect the final amplified product by agarose gel electrophoresis and EtBr staining.

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