An Improved Agar Plating Assay for Detecting *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in Contaminated Bean Seed

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ABSTRACT

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An assay employing semiselective agar media was developed for detection of *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in bean seed. The bacteria were extracted from seed by soaking a 1-kg sample in 3 L of sterile saline with 0.01% Tween 20 for 20 hr at 5 C. *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* were detected by plating 0.1-ml samples of the extract onto triplicate plates of the semiselective agar media and incubating them at room temperature. Two semiselective media were developed: KBC for *P. s.* pv. *syringae* and MSP for *P. s.* pv. *phaseolicola*. Medium KBC, a modification of medium B of King et al (KB) was made by adding boric acid, cephalexin, and cycloheximide to KB. Recovery of 10 strains of *P. s.* pv. *syringae* on KBC ranged from 80 to 139%, and more than 90% of seed-associated saprophytes were inhibited on KBC when compared with KB. As few as 2.1×10^4 colony-forming units (cfu) of *P. s.* pv. *syringae* (from infected bean leaf material) added to a 1-kg seed sample were detected on KBC. Medium MSP was made by adding cephalexin, vancomycin, cycloheximide, and bromthymol blue to sucrose peptone agar. MSP showed plating efficiencies of 87–124% for nine strains of *P. s.* pv. *phaseolicola*, and more than 80% of bean seed-associated saprophytes growing on KB were inhibited. *P. s.* pv. *phaseolicola* was detectable in seed samples containing as few as two naturally contaminated seeds (10^2–10^5 cfu per seed) when plated on MSP. Gravity culled and milled commercial seeds from 14 fields suspected of being infected with *P. s.* pv. *phaseolicola* and/or *P. s.* pv. *syringae* in 1984 were assayed on the two semiselective media and KB; either or both of the pathogens were detected in all 14 lots on KBC or MSP but in only seven lots on KB.

Additional key words: brown spot, halo blight.

Pseudomonas syringae pv. *syringae* van Hall and *P. s.* pv. *phaseolicola* (Burkholder) Young et al, the causal agents of brown spot and halo blight, respectively, are serious pathogens of beans (*Phaseolus vulgaris* L.) and can reduce yield and quality (26,28). Contaminated seed is a source of primary inoculum (13,20) and consequently, production and certification of seed free of these pathogens is an important step in controlling brown spot and halo blight. In the United States, most of the bean seed is grown in the arid region of southern Idaho where bacterial blights are normally not a problem (26). However, in the early 1960s, severe losses occurred from halo blight, and, in response, the Idaho seed industry, through the Idaho State Department of Agriculture, adopted rules requiring field inspections for bacterial blight and laboratory testing of seeds for *P. s.* pv. *phaseolicola* (26). A zero tolerance was established; seed contaminated with blight organisms could not be sown and any field found infested was plowed. The key to the success of a seed health certification program is the availability of a reliable and rapid method to detect contaminated seed lots and to isolate the pathogen from field samples. Several methods have been used for detection of the halo blight and brown spot pathogens in seed, including plating seed soak liquid onto medium B (21,23) of King et al (KB) (8), inoculating bean plants with soak liquid from cull seeds (26), "Dome" test (25), and such direct serological assays as agar gel diffusion (4), immunofluorescence (22), and enzyme-linked immunosorbent assay (ELISA) (23). Field inspections have had limited success and are considered less sensitive than the cull seed soak liquid plant inoculation test which, in turn, is not foolproof (26). The recurrence of severe blight epiphytotics in Idaho in 1977 and 1984 suggests that the control program is not entirely successful. The Asgrow cull seed soak assay (26) is currently being used only by a limited number of seed companies because the test is very time consuming and requires considerable greenhouse space. Although agar plating assays have several advantages over host inoculation or direct serological tests (16), no sensitive selective medium has been available for the brown spot and halo blight pathogens. This paper reports the development of a bean seed assay based on semiselective agar media for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola*. A preliminary report describing the semiselective KBC medium has been published (11).

MATERIALS AND METHODS

Cultures. Strains of *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola*, *P. s.* pv. *pisi*, and *P. s.* pv. *tomato* used in recovery experiments were freshly isolated or obtained from culture collections (Table 1). Throughout the study, all the inoculated plates were incubated at room temperature.

Development of semiselective agar media. Of the media reported to be selective for certain plant pathogenic pseudomonads, the following were evaluated in preliminary tests: BCBRVB (15), D-4 (7), SNR (19), KFT (1), Proline agar (12), and M-71 (10). In addition, 18 antibiotics and nalidixic acid were evaluated by the paper disk method (14); crystal violet (3,27) and boric acid (10) were tested by incorporation into KB. Three strains each of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* were used for these experiments.

Two semiselective media were developed: KBC for *P. s.* pv. *syringae* and MSP for *P. s.* pv. *phaseolicola*. All ingredients mentioned were obtained from Sigma Chemical Co., St. Louis, MO. Medium KBC (a modification of KB) contains boric acid (1.5 mg/ml), cephalexin (80 μg/ml), and cycloheximide (200 μg/ml). To prepare KBC, 900 ml of KB was first made with ingredients for a liter, autoclaved, and cooled to 45 C. Then, 100 ml of a 1.5% (w/v) aqueous solution of boric acid (autoclaved and cooled to 45 C), 8 ml of a 25 mg/ml stock solution of cycloheximide (in 12.5% methanol), and 8 ml of a 10 mg/ml stock solution (aqueous) of cephalexin were added aseptically. Medium MSP, a modification of sucrose peptone agar (6), contains bromthymol blue (15 μg/ml), cephalexin (80 μg/ml), vancomycin (10 μg/ml), and cycloheximide (200 μg/ml). To prepare MSP, 1 ml of a 1.5% (w/v) alcoholic solution (in 20% ethanol) of bromthymol blue was added per liter of sucrose peptone agar (6), pH adjusted to 7.2–7.4, autoclaved, and cooled to 45 C. Then, 1 ml of a 10-mg/ml stock solution (aqueous) of vancomycin, followed by cephalexin and cycloheximide as in KBC, was added.

To determine growth on the final media, 11 strains of *P. s.* pv. *syringae* and nine of *P. s.* pv. *phaseolicola* were tested on KBC and MSP, respectively. Bacteria were grown in liquid medium 523 (7), diluted, and plated (24) onto KB and the respective test medium. All plating efficiencies and inhibition of saprophytes were determined (18) in comparison to KB and were obtained from plates containing 50–200 colonies per plate.

Evaluation of the media for inhibition of bean seed associated saprophytes. One kilogram of seed (approximately 3,000–5,000 seeds, depending on seed lot) was soaked in 3 L of cold (5 C) sterile saline (0.85% NaCl) with 0.01% Tween 20 (SST) in 5-L autoclavable, polyethylene specimen containers (Markson Science, Phoenix, AZ). After 20 hr at 5 C (23), the suspension was stirred thoroughly with a sterile heavy-duty glass rod and a 30-ml sample was removed. Serial 10-fold dilutions were made to 10^{-3} and assayed by plating (24) 0.1-ml samples on three plates each of KB, MSP, and KBC.

Recovery of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* from seed containing natural inoculum. All tests were performed with 1-kg samples (about 3,000 seeds) of a single seed lot of cultivar Viva. This seed lot was chosen because assays failed to detect *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* and more types of saprophytes were observed in comparison to seven other seed lots tested. For inoculum of *P. s.* pv. *syringae*, pulverized leaf material from infected plants (obtained from D. J. Hagedorn, University of Wisconsin-Madison) was used, as described (5). From the 30-g sample, 5 mg was added to 5 ml of cold (5 C) SST in 25-mm-diameter test tubes. After extracting on a rotary shaker for 2 hr at 5 C, 10-fold serial dilutions were made to 10^{-3}, and 0.1 ml of each dilution was plated onto triplicate plates of KB and KBC. The experiment was replicated four times and the mean number of colony-forming units (cfu) of *P. s.* pv. *syringae* per milligram of leaf material was determined. Sufficient leaf material (6 mg), based on the above results, was then added to each of three specimen containers with 3 L of SST and 1 kg of seeds to result in a final expected population of approximately 0.5 cfu of *P. s.* pv. *syringae* per milliliter (1.5×10^3 cfu per 3 L). The contents were mixed and incubated in a walk-in cold room at 5 C. After 2 and 20 hr, the seeds were thoroughly mixed with a sterile glass rod and 150-ml samples were drawn from each container. Each sample was concentrated 100-fold by centrifugation at 12,000 *g* for 10 min and by resuspending the pellet in 1.5 ml of sterile saline, and 0.1-ml samples were plated onto KB and KBC as described earlier.

For *P. s.* pv. *phaseolicola*, 80 seeds of cultivar Charlevoix harvested from infected plants (obtained from A. W. Saettler, Michigan State University) were used. To determine the frequency of contaminated seeds and the range of colony-forming units of *P. s.* pv. *phaseolicola* per seed, 17 seeds of similar appearance were selected. Eleven of these 17, chosen at random, were then placed singly in 25-mm-diameter test tubes containing 5 ml of cold (5 C) SST. The tubes were shaken on a rotary shaker for 2 hr at 5 C. The number of colony-forming units per seed was determined by plating 0.1 ml of 10-fold dilutions of the suspension onto three plates each of KB and MSP. Of the remaining six seeds, two were added to each of three replicates of 1 kg of seed (cultivar Viva) in 3 L of SST. The seeds were extracted and sampled as described for *P. s.* pv. *syringae* above and 0.1 ml of undiluted, 10-fold diluted, and 10-fold concentrated samples plated onto triplicate plates of KB and MSP.

Assay of commercial seed lots. To determine the effectiveness of the extraction procedure and the semiselective media in recovering *P. s.* pv. *syringae* and/or *P. s.* pv. *phaseolicola* from naturally contaminated seeds, normal milled and gravity culled seeds of 14 lots were obtained. These seed lots had been found to be contaminated with *P. s.* pv. *syringae* and/or *P. s.* pv. *phaseolicola* in the Asgrow soak assay (26). This assay involves soaking 25 kg of cull seed for 24 hr and inoculation of bean seedlings with the soak liquid. No data were available on the possible contamination of milled seeds from these lots. Samples of 1 kg (3,000–5,000 seeds) of milled seed were assayed as described previously. If either *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* was detected in the milled seed, no cull seed samples of that seed lot were assayed. However, if the pathogens were not detected in milled seeds, a sample of cull seed from the same lot was assayed. For milled seeds, 0.1 ml of original, 10-fold diluted, and 10-fold concentrated soak liquid was plated onto triplicate plates of KB, KBC, and MSP. The 10-fold concentrated sample was stored at 3–5 C. If no colonies of *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* were found and if only few saprophytes were observed in the 10-fold concentrated sample, the sample was further concentrated 10-fold in a micro centrifuge (Eppendorf model 5414, VWR Scientific, San Francisco, CA) and plated as above. For cull seeds, 0.1 ml of undiluted and 10-, 100-, and 1,000-fold dilutions of soak liquid were plated as above. Total number of colonies showing characters typical of *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* were recorded.

Identification of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* colonies. Representative colonies, presumptively considered as *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola*, based on morphological characteristics like size, color, consistency, elevation, and presence of pale-blue fluorescent pigment, were purified by streaking onto KB plates and identified by oxidase reaction, levan formation, production of acid from mannitol and erythritol (15), and pathogenicity tests. For pathogenicity tests, cultures were grown in medium 523 overnight and adjusted to 10^7 cfu/ml (15). Leaves of 10–12-day-old plants of Bush Blue Lake No. 7 beans were inoculated as described (9), placed in a dew chamber (Percival model E-54U-b1, Percival, Boone, IA) at 22 C for 48 hr, and then in a growth chamber at 25 C for 4 or 5 days.

RESULTS

Development of semiselective agar media. Preliminary evaluation of some media previously reported to be selective for plant pathogenic pseudomonads (BCBRVB, D-4, SNR, KFT, Proline agar, and M-71) resulted either in poor recovery of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* or failed to adequately inhibit the growth of saprophytic flora from bean seed (results not presented). On the other hand, the two new semiselective media, KBC for *P. s.* pv. *syringae* and MSP for *P. s.* pv. *phaseolicola*, permitted good recovery of the respective pathogen (Table 1) and inhibited growth of more than 80% of saprophytic bacteria commonly associated with bean seed (Table 2).

Medium KBC for *P. s.* pv. *syringae*. Plating efficiencies for 10 strains of *P. s.* pv. *syringae* from bean varied from 80 to 139% with a mean of 105% (Table 1). Colonies of *P. s.* pv. *syringae* were 1.5–2.0 mm in diameter after 2 days at room temperature. After 3 days, colonies were 3–3.5 mm in diameter, flat, circular, translucent, creamy white, and showed blue fluorescence under UV light. One strain of *P. s.* pv. *syringae* from pea, three of *P. s.* pv. *pisi*, and one of *P. s.* pv. *tomato* grew well also (Table 1). Growth of bean seed associated saprophytic bacteria on KBC was reduced significantly (more than 90%), compared with KB (Table 2).

Medium MSP for *P. s.* pv. *phaseolicola*. Plating efficiencies of nine strains of *P. s.* pv. *phaseolicola* ranged from 87 to 124% with a mean of 102% (Table 1). Colonies of *P. s.* pv. *phaseolicola* were

1.5–2.0 mm in diameter after 2 days and 3 mm after 3 days at room temperature. They were circular, raised, globose, glistening, and light yellow with a less dense center. The medium around the colony turned light yellow after 3 days. Growth of saprophytic bacteria from bean seed washings was reduced significantly (more than 80%), compared with KB (Table 2).

Crystal violet inhibited growth of *P. s.* pv. *phaseolicola* at concentrations of 30 (27) and 300 (3) mg/ml. *P. s.* pv. *phaseolicola* grew in the presence of 3 mg of crystal violet/ml, but recovery, compared with KB, ranged from a low of 9 to a high of 69% depending on the strain. Boric acid, although well tolerated by *P. s.* pv. *syringae*, was inhibitory to *P. s.* pv. *phaseolicola* (Table 1).

Recovery of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* from seed containing natural inoculum. Approximately 230 cfu of *P. s.* pv. *syringae*, added to 1 kg of seed and extracted in 3 L of SST, could be detected on KBC but not on KB (Table 3). For *P. s.* pv. *phaseolicola*, three of the 11 suspected contaminated individual seeds assayed actually resulted in the recovery of the pathogen. Numbers of colony-forming units of *P. s.* pv. *phaseolicola* per seed were 1.1×10^2, 4.4×10^3, and 3.0×10^5. *P. s.* pv. *phaseolicola* was isolated in two of the three replications of 1 kg of seed, each containing two suspected contaminated seeds (Table 4).

Assay of commercial seed lots. Results of plating the extract from a typical commercial seed lot are shown in Figure 1. Very few saprophytic bacteria grow on KBC; *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* can easily be differentiated from a majority of those saprophytic bacteria able to grow on MSP, based on colony morphology and color. *P. s.* pv. *syringae* and/or *P. s.* pv. *phaseolicola* were isolated from eight of the 14 samples of milled seed (Table 5). Neither pathogen was detected in two of those eight contaminated seed lots (103 and 108) in the first assay. However, when extracts from these two lots were concentrated further (to 100-fold) and reassayed, both pathogens were detected. These pathogens were detected from eight of the seed lots on semiselective media, whereas they could be recovered from only four seed lots on KB. Nine cull seed lots were assayed and *P. s.* pv. *syringae* and/or *P. s.* pv. *phaseolicola* were detected from all the nine on semiselective media, but from only four on KB (Table 6). From seed lots 106 and 110, *P. s.* pv. *syringae* was detected only on KBC. Populations recovered from cull seed lots ranged from 6.6×10^1 per milliliter (seed lot 111, on KBC) to 2.3×10^6 per milliliter (seed lot 104, on MSP).

P. s. pv. *syringae* was isolated from five out of 14 milled seed lots and from all the nine cull seed lots tested, whereas *P. s.* pv. *phaseolicola* was isolated from four milled and only one cull lot.

DISCUSSION

Although semiselective agar media have been useful for detection and presumptive identification of certain seedborne bacteria (17), none has been available for *P. s.* pv. *syringae* or *P. s.* pv. *phaseolicola* on beans. Serological techniques, such as agar gel diffusion (4), immunofluorescence (22), and ELISA (23), have been used for routine detection of these pathogens in seeds. However, besides aspects of specificity and sensitivity, they have the distinct disadvantage of not being able to provide information on viability and pathogenicity (16) of the seedborne inoculum. With an organism such as *P. s.* pv. *syringae*, which can attack several different hosts, data on pathogenicity could be very important.

Plating extracts on KB has been used previously to assay bean seed for *P. s.* pv. *phaseolicola*, but with limited success (21,23). KB worked well with those seed lots containing few saprophytes and large numbers of *P. s.* pv. *phaseolicola* but not with those having large numbers of saprophytes and few *P. s.* pv. *phaseolicola*. The major advantage of the semiselective agar media over KB is the significant reduction in saprophytes.

The ability of a plant pathogenic bacterium to grow in the presence of boric acid was first recognized by Leben (10); medium M-71 contains boric acid and was designed for isolating *P. s.* pv.

TABLE 1. Recovery of strains of different pathovars of *Pseudomonas syringae* on the semiselective media compared to medium B of King et al (KB)

Laboratory ID no.	Pathovar	Strain	Source[y]	Host	Mean recovery (%)[w,x]	
					KBC	MSP
C-282	*syringae*	BS-10-84	1	Bean	139 a	ND[z]
C-289	*syringae*	Original	9	Bean	118 b	ND
C-229	*syringae*	P1	2	Bean	114 bcd	ND
C-283	*syringae*	4-1	3	Bean	107 bcde	ND
C-203	*syringae*	NA6NDA-29	1	Bean	101 bcdef	ND
C-275	*syringae*	BS-3-84	1	Bean	100 bcdef	ND
C-269	*syringae*	PS-3	4	Bean	97 cdefg	ND
C-228	*syringae*	B-3793	2	Bean	96 defg	ND
C-271	*syringae*	PS-17	4	Bean	94 efg	ND
C-276	*syringae*	BS-4-84	1	Bean	80 gh	ND
C-205	*syringae*	239-A	5	Pea	98 cdef	ND
C-214	*pisi*	870A	5	Pea	134 a	ND
C-211	*pisi*	862A	5	Pea	115 bc	ND
C-213	*pisi*	203	5	Pea	113 bcd	ND
C-171	*tomato*	Original	9	Tomato	104 bcdef	ND
C-267	*phaseolicola*	Original	8	Bean	47 i	124 a
C-199	*phaseolicola*	Brege	6	Bean	72 h	112 b
C-206	*phaseolicola*	882	5	Bean	0 k	109 bc
C-290	*phaseolicola*	Original	9	Bean	ND	104 c
C-235	*phaseolicola*	NCPPB 1104	7	Bean	19 j	97 d
C-251	*phaseolicola*	HB-16	6	Bean	2 k	96 d
C-200	*phaseolicola*	DM-123	6	Bean	38 i	96 d
C-226	*phaseolicola*	701A	5	Bean	ND	88 e
C-233	*phaseolicola*	R2	1	Bean	87 fgh	87 e

[w] Recovery from suspension in sterile 0.85% NaCl. Mean recovery = (number of colonies recovered on semiselective medium × 100)/(number of colonies on KB). Figures are calculated from the mean number of colonies per plate, three plates per strain. Mean percentage values in a column followed by the same letter are not significantly different ($P = 0.01$) by Duncan's multiple range test.

[x] KBC: KB plus boric acid, cephalexin, and cycloheximide; MSP: modified sucrose peptone agar with bromthymol blue, cephalexin, vancomycin, and cycloheximide.

[y] Source names and location: 1 = D. M. Webster, Twin Falls, ID; 2 = D. J. Hagedorn, Madison, WI; 3 = L. Nadolny, Boise, ID; 4 = J. P. Hubbard, Farmington, WI; 5 = J. D. Taylor, Wellesbourne, England; 6 = J. W. Guthrie, Moscow, ID; 7 = R. Lelliott, National Collection of Plant Pathogenic Bacteria, Harpenden, England; 8 = H. S. Fenwick, Moscow, ID; and 9 = authors.

[z] ND = Not determined.

glycinea. The high selectivity of KBC medium for *P. s.* pv. *syringae* is due primarily to boric acid (11). Washings from many seed lots tested could be concentrated 100-fold for plating onto KBC. This allowed for the detection of as few as 2.1×10^4 cfu of *P. s.* pv. *syringae* contained in 1 kg of bean seed. In addition to seed assays, the medium has been useful in isolating *P. s.* pv. *syringae* from bean and alfalfa plant material from southern Idaho (*unpublished*). The high plating efficiencies of *P. s.* pv. *pisi* and *P. s.* pv. *tomato* on KBC indicate that this medium might also be useful in assaying pea and tomato seeds for these pathogens.

Although MSP is less selective for *P. s.* pv. *phaseolicola* than KBC is for *P. s.* pv. *syringae*, MSP represents a distinct improvement over KB. MSP not only results in a significant reduction in saprophytic bacteria, but also allows relatively easy differentiation of *P. s.* pv. *phaseolicola* from those saprophytes able to grow on this medium, based on production of levan and acid from sucrose in the presence of bromthymol blue. Because strains of *P. s.* pv. *phaseolicola* either cannot grow or grow very slowly on KBC, plating seed extracts on both KBC and MSP might indicate the identity of the pathogen involved, especially if typical pathogen colonies are detected only on MSP.

Assay of bean seed obtained from plants naturally infected with *P. s.* pv. *phaseolicola* shows that all seeds are not necessarily contaminated, and even among the contaminated seeds, the level of contamination varies widely. These observations agree with those of Taylor et al (21). However, the mean level of contamination of the seed we tested is much lower than the level (3.4×10^7 cfu per seed) reported by Taylor et al (21) for severely infected seeds. These results point to the necessity of considering the actual range of pathogen colony-forming units carried by the contaminated seed and not the number of added infected seeds per se in establishing the sensitivity of the detection methods.

Whereas these semiselective media improve the chances of successful isolation of the pathogen, they are not of definitive diagnostic value. Improvements in methods of extraction of the pathogen from the seed (21,23) coupled with rapid and specific means of identification (17,23) of the bacterium isolated on the medium will be necessary for more efficient and reliable seed assays. For example, a highly specific DNA probe (17) is being tested for rapid identification of the suspected *P. s.* pv. *phaseolicola* colonies growing on MSP in the bean seed assay. The failure to detect the pathogens in six of the milled commercial seed samples tested in this study illustrates the need for testing more

TABLE 2. Reduction in population of saprophytic bacteria isolated from different lots of bean seed on semiselective agar media relative to medium B of King et al (KB)[v]

Seed lot	Mean number of colonies of saprophytic bacteria $\times 10^{-2}$/ml of extract		
	KB	MSP[w]	KBC[x]
Viva	93.4 a[y]	18.2 b (80.5)[z]	0.7 c (99.3)
Lake Superior 435	232.0 a	17.3 b (92.5)	2.0 c (99.1)
Green Crop 136	161.3 a	32.7 b (79.7)	4.7 c (97.1)
Triumph 201	181.7 a	24.0 b (86.8)	4.1 c (97.7)
106	46.7 a	1.3 b (97.2)	0.5 b (98.9)
101	86.3 a	4.0 b (95.4)	7.2 b (91.7)
109	38.3 a	2.0 b (94.8)	0.2 b (99.5)
113	257.0 a	12.3 b (95.2)	10.3 b (96.0)

[v] One kilogram of seed (3,000–5,000 seeds) was added to 3 L of cold sterile saline with 0.01% Tween 20 in 5-L containers. After 20 hr at 5 C, the seeds were stirred and a 30-ml sample of liquid was removed and assayed by plating 0.1 ml of serial 10-fold dilutions on triplicate plates of each medium.

[w] MSP: Modified sucrose peptone medium with bromthymol blue, cephalexin, vancomycin, and cycloheximide.

[x] KBC: KB plus boric acid, cephalexin, and cycloheximide.

[y] Figures followed by the same letter in each row (for each seed lot) are not significantly different (FLSD, $P = 0.01$).

[z] Figures in parentheses are percent reduction determined as follows: 100-(number of saprophytic bacterial colonies observed on semiselective medium per milliliter of seed washing $\times$ 100)/number of saprophytic bacterial colonies observed on KB per milliliter of seed washing.

TABLE 3. Recovery of *Pseudomonas syringae* pv. *syringae* from bean seed mixed with inoculum of naturally infected bean leaves, on medium B of King et al (KB) and semiselective agar medium KBC[y]

Mean number of cfu/ml of check[z]	Mean number of cfu/ml after soaking seeds for			
	2 hr		20 hr	
	KB	KBC	KB	KBC
0.23	0.0	0.47	0.0	0.44

[y] Six milligrams of bean leaf powder naturally infected with *P. s.* pv. *syringae* (expected to contain 2.1×10^4 cfu) was added to each of the three replicates of 1 kg of seed (cultivar Viva) in 3 L of sterile saline plus 0.01% Tween 20 (SST) and incubated at 5 C. Samples (150 ml) were concentrated 100-fold by centrifuging at 12,000 *g* for 10 min and suspending the pellet in 1.5 ml of sterile saline, and 0.1 ml of suspension was spread on three plates each of KB and KBC.

[z] Checks consisted of three replicates of 3 L of SST (without bean seed), to which 6 mg of infected leaf powder was added, and were incubated, sampled, and assayed as above.

TABLE 4. Recovery of *Pseudomonas syringae* pv. *phaseolicola* from bean seed samples containing naturally contaminated seeds, on medium B of King et al (KB) and semiselective agar medium MSP[z]

Replicate	Mean number of cfu/ml after soaking seeds for			
	2 hr		20 hr	
	KB	MSP	KB	MSP
1	1.0×10^2	1.0×10^2	3.3×10^2	1.7×10^2
2	0.0	3.3×10^1	1.3×10^2	1.3×10^2
3	0.0	0.0	0.0	0.0

[z] Two seeds from the naturally contaminated lot were added to each sample of 1 kg of seed (cultivar Viva) in 3 L of sterile saline plus 0.01% Tween 20 and incubated at 5 C. Aliquots of 0.1 ml of undiluted, 10-fold diluted, and 10-fold concentrated samples were plated onto triplicate plates of KB and MSP (a semiselective medium for *P. s.* pv. *phaseolicola*).

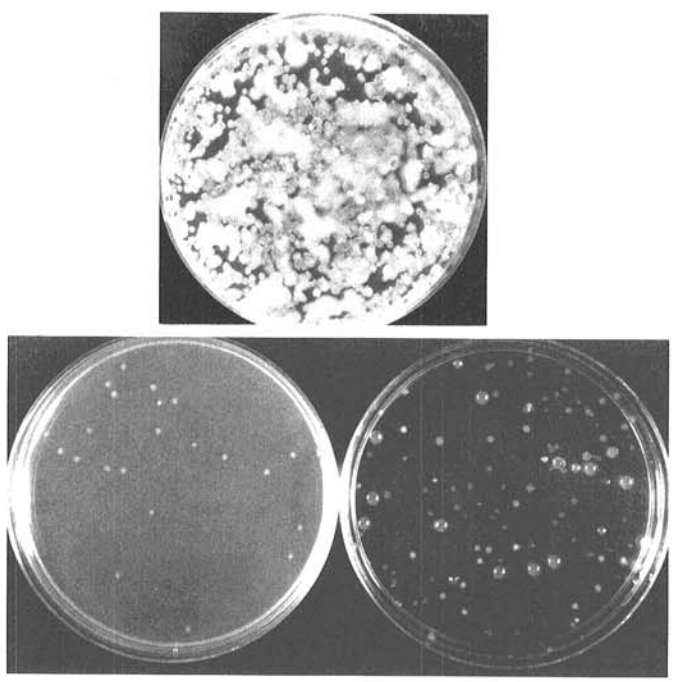

Fig. 1. Reduction of numbers of bean seed-associated saprophytes and recovery of *Pseudomonas syringae* pv. *syringae* from a naturally contaminated commercial seed lot on semiselective media KBC (lower left) and MSP (lower right), compared with KB (top center). Note the raised colonies of the pathogen on MSP.

TABLE 5. Detection of *Pseudomonas syringae* pv. *syringae* or *P. s.* pv. *phaseolicola* in milled commercial bean seeds[w,x]

Seed lot	KB (mean cfu)	KBC (mean cfu)	MSP (mean cfu)	Pathogen[y] identity
102	500	0	500	Psp
103	0	0.022[z]	0	Pss
104	0	0	0	...
105	0	0	0	...
106	0	0	0	...
107	0	0	1,400	Psp
108	0	0.60[z]	0.30[z]	Pss/Psp
109	0	0	0	...
110	0	0	0	...
111	0	0	0	...
112	2	0	2	Psp
113	0	1,000	31	Pss
114	510	1,300	420	Pss
115	190	160	160	Pss

[w] One-kilogram samples of seed (3,000–5,000 seeds) were extracted for 20 hr at 5 C in 3 L of sterile saline + 0.01% Tween 20. One-tenth milliliter of undiluted, 10-fold diluted, and 10-fold concentrated (by centrifugation) extracts were pietted onto three plates each of medium B of King et al (KB), and the semiselective media KBC (for *P. s.* pv. *syringae*) and MSP (for *P. s.* pv. *phaseolicola*.

[x] Data presented as mean number colony-forming units $\times 10^{-1}$ ml of extract.

[y] Pss = *P. s.* pv. *syringae*; Psp = *P. s.* pv. *phaseolicola*.

[z] Detected only by plating 100-fold concentrated extract.

TABLE 6. Mean number of colony-forming units (cfu)/ml of *Pseudomonas syringae* pv. *syringae* or *P. s.* pv. *phaseolicola* recovered from cull bean seeds, on medium B of King et al (KB) and the semiselective agar media[y]

Seed lot	KB	KBC	MSP	Pathogen identity[z]
102	9.3×10^4	2.3×10^4	1.9×10^5	Pss/Psp
103	0	1.7×10^2	1.0×10^5	Pss
104	1.8×10^6	5.4×10^5	2.3×10^6	Pss
105	0	4.0×10^4	3.0×10^4	Pss
106	0	1.1×10^4	0	Pss
107	4.7×10^4	6.1×10^4	4.6×10^4	Pss
109	1.0×10^5	2.2×10^5	1.7×10^5	Pss
110	0	2.7×10^3	0	Pss
111	0	6.6×10^1	1.3×10^2	Pss

[y] One-kilogram samples of seed (3,000–5,000 seeds) were extracted for 20 hr at 5 C in 3 L of sterile saline + 0.01% Tween 20 one-tenth millilter of undiluted, 10-, 100-, and 1,000-fold dilutions of extract were pipetted onto three plates each of KB and the semiselective media KBC (for *P. s.* pv. *syringae*) and MSP (for *P. s.* pv. *phaseolicola*). Figures are calculated from the mean number of cfu per plate.

[z] Pss = *P. s.* pv. *syringae*; Psp = *P. s.* pv. *phaseolicola*.

and/or larger samples of seeds (2,20) or the use of cull seed, as suggested (26).

The following method is recommended for assaying bean seed for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola*. Three 1-kg samples of seed are soaked in 3 L of SST of 5 C for 20 hr (stationary cold extraction). Each suspension is thoroughly mixed with a sterile glass rod and 150-ml sample of the extract is drawn. A 100-ml portion of this extract is concentrated 10-fold by centrifugation at 12,000 *g* for 10 min and suspending the pellet in 10 ml sterile saline (0.85% NaCl). Aliquots of 0.1 ml of 10-fold diluted, undiluted, and 10-fold concentrated extracts are plated onto triplicate plates of KBC and MSP and incubated at room temperature (23 ± 2 C) for 3 or 4 days. The 10-fold concentrated sample is stored at 3–5 C. If no typical colonies of either of the pathogen are detected and if only few saprophyte colonies are observed in the 10-fold concentrated sample, this sample was concentrated 10-fold more in a micro centrifuge and plated as above. Representative colonies with characteristics typical of *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* (described above for each medium) are purified by streaking onto KB plates and identified by appropriate bacteriological (15) and pathogenicity tests.

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