Modified Bean Pod Assay to Detect Strains of *Pseudomonas syringae* pv. *syringae* That Cause Bacterial Brown Spot of Snap Bean

G. Y. CHENG, D. E. LEGARD, J. E. HUNTER, and T. J. BURR, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456

ABSTRACT

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Approximately 99% of 1,310 strains of *Pseudomonas syringae* pv. *syringae* isolated from bacterial brown spot (BBS) lesions on beans in New York State, Wisconsin, and Colorado caused a unique symptom when assayed on excised bean pods. This symptom could be distinguished from those caused by 45 of 46 strains of *P. s.* pv. *syringae* recovered from 15 other crop species. The exception was a strain isolated from almond in California, which caused symptoms indistinguishable from those caused by strains from BBS lesions. None of 68 strains of 17 other pathovars of *P. syringae*, including 34 strains of *P. s.* pv. *phaseolicola*, or eight strains of *P. viridiflava* incited symptoms that could be confused with those caused by strains of *P. s.* pv. *syringae* recovered from BBS lesions. Of 337 epiphytic strains of *P. syringae* recovered from leaves of 51 weed species, 23 strains incited symptoms on detached pods that were identical to those caused by strains from BBS lesions. These strains were recovered only from sites where BBS occurred that year. Consistent results were obtained with a procedure that involves placing a smear of bacterial cells on the pod surface and inserting an insect-mounting pin through the cells and laterally underneath the epidermis of greenhouse-grown snap bean pods (cultivar Bush Blue Lake 274). Since the procedure does not require calculated inoculum concentrations, it is useful in evaluating large numbers of strains (e.g., epiphyte collections) for their ability to cause BBS. Pod assays were more rapid and reliable than leaf assays of whole plants in distinguishing strains of *P. s.* pv. *syringae* capable of causing BBS.

The first observation of bacterial brown spot (BBS) of bean (*Phaseolus vulgaris*), caused by *Pseudomonas syringae* pv. *syringae* (3,7,9), was by Burkholder (3) in 1930, in New Jersey. The disease remained rare in the United States for many years (30). In 1960 BBS was observed in Wisconsin, and by 1963 it had become serious (24). Since then it has been found in other bean-producing states (28). The symptoms on leaves are necrotic, brown lesions, sometimes with marginal chlorosis, but lacking halos. Pods are often characteristically bent at the site of a lesion (12,24), although this symptom was not reported by Burkholder (3) in his original description of the disease.

Genetically diverse phytopathogenic bacteria were reported to incite characteristic symptoms in inoculated bean pods (17). Saad and Hagedorn (27) inoculated bean pods to compare strains of *P. s.* pv. *syringae* from different hosts. They found that strains from beans caused water-soaked lesions without any brown necrosis on inoculated pods, whereas strains from other sources caused brown, necrotic, sunken lesions. Ercolani et al (10) divided strains of *P. syringae* into two groups on the basis of symptoms that developed after pods were injected with a droplet of inoculum containing 10^6–10^8 cfu/ml. One group of strains caused sunken, dry, necrotic, dark brown lesions after 2 days; the other group caused green, water-soaked, sunken lesions within 3 days. The first group had been isolated from several crop species, and the second recovered from bean, hairy vetch, and yellow sweet clover. Genetic diversity among strains of *P. s.* pv. *syringae* was also indicated by bean pod assays in several other BBS studies (8,21,29) and data from two serological studies (11,22). These results are consistent with Bradbury's (2) description of *P. s.* pv. *syringae*, as "a complicated mosaic of slightly different taxa from a large number of different host plants."

BBS has become increasingly prevalent in snap beans in New York State since it was observed in 1980. It first appeared in areas where *P. s.* pv. *syringae* has occurred commonly on fruit crops since 1946 (5,23) and where snap beans have been grown free of BBS for many years. Since BBS was not observed before 1980, it was postulated that strains causing BBS are different from those associated with fruit crops. Consequently, a study was initiated to determine the etiology of BBS in New York State. We report evidence that strains of *P. s.* pv. *syringae* isolated from BBS lesions are different from those recovered from other sources, as determined by a bean pod assay that was modified to facilitate the rapid testing of large numbers of strains. A preliminary report of this study has been published (6).

MATERIALS AND METHODS

A selective medium (PSM) developed by Burr and Katz (4) was modified to make colonies of *P. s.* pv. *syringae* easier to identify. The composition of the modified medium (mPSM), per liter, is as follows: peptone type III (Sigma), 20 g; glycerol (Sigma), 9 ml; sodium taurocholate (Difco), 3 g; K_2HPO_4 (Sigma), 1.5 g; $MgSO_4$ (Sigma), 1.5 g; Tergitol 7 (Sigma), 0.1 ml; and agar (Difco), 15 g. Novobiocin (Sigma) and cycloheximide (Sigma) were filter-sterilized and added to cooled medium (45 C) to final concentrations of 50 and 100 mg/L, respectively.

Two strains of *P. s.* pv. *syringae* isolated from apple (Pss-7) and cherry (Pss-24) and two strains from BBS lesions on snap bean (Pss-18 and CB-6) were used to evaluate inoculation procedures. Freshly excised snap bean pods that were 8–10 cm long, in the early stage of seed formation, were collected from greenhouse-grown plants (cultivar Bush Blue Lake 274) and washed in 0.13% hypochlorite for about 10 sec. They were then rinsed twice with tap water and blotted dry on a paper towel in a laminar flow hood.

Three inoculation procedures were compared: 1) a bacterial suspension (about 10^8 cfu/ml) was injected subepidermally according to the procedure of Klement and Lovrekovich (17); 2) bacterial cells removed from a nutrient agar plate with a toothpick were placed on the surface of a pod, and a number 2 insect-mounting pin was pushed through the cells and inserted perpendicularly through the epidermis for about 2 mm; and 3) a pin similarly coated with bacterial cells was inserted laterally underneath the epidermis for about 4 mm. For each strain of bacteria, three pods were inoculated at four to six sites (about 1 cm apart) per pod. Excess inoculum was removed from the pod surface with the pin and used to inoculate replicate sites. On each pod, a control inoculation was performed without bacteria. Inoculated pods were placed on a wire rack covered with a moistened paper towel in a closed 23- by 31-cm plastic crisper box containing 1–2 cm of water. They were incubated at room temperature (24–28 C) under fluorescent lights for about 10 hr/day. Symptoms were noted daily for 5 days.

The lateral inoculation procedure

Present address of first author: Department of Plant Protection, Huazhong Agricultural College, Wuhan, Hubei, People's Republic of China.

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described above (procedure 3) was used to inoculate pods to determine variation in symptoms caused by various strains of *P. s.* pv. *syringae*, other pathovars of *P. syringae*, and *P. viridiflava*. As a positive control, each pod was inoculated with a BBS strain known to cause a typical BBS reaction, i.e., green, water-soaked, sunken lesions persisting for at least 4 days without necrosis. Over a 2-yr period, 1,310 strains of *P. s.* pv. *syringae*, isolated from 75 bean samples with BBS, were evaluated by this procedure (Table 1). Also, strains of *P. s.* pv. *syringae* recovered from BBS lesions independently by others were tested: Pss-3, Pss-4, Pss-5, and Pss-27, from J. Hunter, in New York; A1, A18, 48, 441, 515, 795, 888, 900, and 43335, from R. Gilbertson, in Wisconsin; F84-18 and F84-81, from H. Schwartz, in Colorado; and R-32, from K. Rudolph, in West Germany. Forty-six strains of *P. s.* pv. *syringae* recovered from 15 cultivated plant species other than bean were also assayed (Table 2). In addition, symptoms caused by 68 strains of 17 other pathovars of *P. syringae* and eight strains of *P. viridiflava* (Table 2) were compared with those caused by strains of *P. s.* pv. *syringae* recovered from BBS lesions.

Isolations were made from fresh BBS lesions within 24 hr after snap bean samples were collected in New York State or as soon as they were received by overnight air shipment from other locations. Leaves or pods were washed in running tap water, placed in a 0.5% sodium hypochlorite solution for 1 min, and briefly rinsed in sterile distilled water. Small pieces of tissue adjacent to lesions were then macerated in sterile distilled water, and the suspension was streaked onto King's B (16) or mPSM.

For the recovery of strains from epiphytes, 20 g of leaf tissue was placed in a 1,000-ml Erlenmeyer flask containing 200 ml of sterile 0.05 M phosphate buffer, pH 7.0. The flask was agitated with a wrist-action shaker for 2 hr. The resulting suspension was serially diluted in sterile phosphate buffer, and 0.1-ml samples were spread onto mPSM in petri dishes. These were incubated at 22 C for 72 hr. Colonies were examined under long-wave UV light for fluorescence. Presumptive identification of strains as *P. s.* pv. *syringae* was based on positive tests for fluorescence and levan production (19) and a negative test for oxidase (18). Some strains collected in 1986 and all collected in 1987 were subjected to carbon utilization tests (1,19): mannitol, L-tartrate, homoserine, and sorbitol in 1986 and mannitol, inositol, L-tartrate, homoserine, L-lactate, and anthranilic acid in 1987. Additionally, all strains were tested for acid production from sucrose (19) and ice nucleation activity at −3.5 C in 1986 and −3.0 C in 1987 (13). Cultures were stored on nutrient agar medium at 4 C and grown on fresh nutrient agar or King's B for 24–48 hr at 22 C prior to being used for inoculation.

Fluorescent pseudomonads recovered from leaves of various plants were also compared to strains from BBS lesions with the pod assay. The strains were isolated on mPSM from leaf washes of 51 weed species in six locations in central New York State (two fields where BBS had occurred that year, one where beans had been grown the previous year without evidence of BBS, and three from uncultivated areas); 337 strains that were fluorescent, oxidase-negative, and levan-positive were putatively identified as *P. syringae*. Excised pods were inoculated with these strains by the lateral inoculation procedure.

To test the hypothesis that strains of *P. s.* pv. *syringae* causing characteristic symptoms on pods also cause unique symptoms on bean leaves, selected strains recovered from bean leaf washings and BBS lesions were adjusted to a concentration of 10^8 cfu/ml (OD 0.1, determined spectrophotometrically at 620 nm) in phosphate buffer and sprayed onto 4- to 5-wk-old bean plants (cultivar Bush Blue Lake 274) in a greenhouse at 22–25 C, by the procedure of Saad and Hagedorn (26). Strains that caused the BBS-type pod reaction were compared to strains that caused necrotic lesions within 4 days (non-BBS-type pod reaction). Twelve strains were used, and the experiment was repeated three times. Foliar symptoms were assessed 7–10 days after inoculation.

A second leaf inoculation experiment was conducted with 20 randomly selected strains of *P. s.* pv. *syringae* recovered from bean and weed leaf washes: 10 strains that caused the BBS-type pod reaction and 10 that did not. Four strains recovered from BBS lesions in New York, Wisconsin, Colorado, and Germany were included as controls. The cultivars Bush Blue Lake 274 and Bush Blue Lake 92 were inoculated as in the previous experiment. The plants were incubated in a greenhouse at 27–29 C, and symptoms were scored 4, 7, and 10 days after inoculation. Bacteria were isolated from lesions, characterized as previously described, and reassayed on detached pods.

RESULTS

The injection of a suspension of bacterial cells (10^8 cfu/ml) subepidermally into excised bean pods caused distinct and reproducible symptoms, but the preparation of inocula was time-consuming. The insertion of a pin laden with bacterial cells perpendicularly through the epidermis caused the development of a small lesion, which was not optimum for discerning slight but important differences in symptoms. When bacterial cells were inserted with a pin laterally underneath the epidermis for about 4 mm, a large lesion (5–6 cm in diameter) developed. With this third procedure, consistent results were obtained for each strain when the inoculated pods were of uniform size and age.

There was little variation in pod symptoms induced by strains isolated from BBS lesions. All 16 strains from New York, Wisconsin, Colorado, and West Germany and all except 10 of 1,310 strains that we isolated from BBS lesions on beans grown in New York, Wisconsin, and Colorado caused the BBS-type pod reaction, i.e., green, water-soaked, sunken lesions that remained free of necrosis for at least 4 days. Of 46 strains of *P. s.* pv. *syringae* originally recovered from 15 crop species other than bean,

Table 1. Strains of *Pseudomonas syringae* pv. *syringae* isolated from bacterial brown spot (BBS) lesions on bean in 1986 and 1987 and reactions they caused on excised bean pods (cultivar Bush Blue Lake 274)

State where strains were collected	Type of bean[a]	Number of samples collected	Number of strains tested[b]	Number of non-BBS-type strains[c]
New York	BBL 92	16	306	3
	Tenderlake	16	289	1
	BBL 47	6	91	0
	Vitagreen	3	76	4
	Bonanza	4	60	0
	Early Gallatin	3	56	0
	Labrador	3	48	0
	Golden Rod	2	48	0
	Dandy	3	44	0
	Burley	2	36	0
	Strike	2	28	0
	Eagle	2	24	0
	Bee-Line	1	12	0
	Snap bean	3	48	0
Wisconsin	Snap bean	6	96	2
Colorado	Dry bean	3	48	0
Totals		75	1,310	10

[a] *Phaseolus vulgaris*; all named cultivars are snap bean. BBL = Bush Blue Lake.
[b] Assayed on excised pods by pin inoculation.
[c] Strains that did not cause a BBS-type pod reaction.

only one strain (B-15+, from almond in California) caused the BBS-type pod reaction, although some strains caused green, water-soaked, sunken lesions without obvious necrosis for 2–3 days. None of 68 strains of 17 other pathovars of *P. syringae* caused symptoms typical of those caused by BBS strains. With the exception of *P. s.* pv. *phaseolicola*, the causal agent of halo blight of bean, all caused a necrotic reaction relatively quickly. Lesions incited by *P. s.* pv. *phaseolicola* were similar to those caused by the BBS pathogen but were not sunken. This difference was confirmed with 34 strains of *P. s.* pv. *phaseolicola* obtained from six states (Table 2). Eight strains of *P. viridiflava*, another bean pathogen (30), caused rust-colored lesions within 48 hr on excised pods, which were easily distinguished from lesions caused by *P. s.* pv. *syringae* (Table 3).

Of 337 strains of *P. syringae* recovered from weeds, only 23 caused the BBS-type pod reaction (Table 3). These strains came from weeds that were collected in or adjacent to fields where BBS was severe that year. Ice nucleation and carbon utilization tests confirmed that all strains that caused the BBS-type pod reaction were *P. s.* pv. *syringae*.

With the exception of the strain from almond, all pathogenic strains of *P. s.* pv. *syringae* from hosts other than bean caused necrosis at the point where the inoculation pin had been inserted. Some water-soaking was apparent initially, but the lesion became necrotic within 1–3 days. Strains of *P. syringae* from weeds in areas where BBS had not occurred caused extensive necrosis within 24 hr without water-soaking.

Strains that caused the BBS-type pod reaction incited BBS symptoms on leaves in the greenhouse (Table 4), and they often caused a puckering of leaf tissue around the lesions. Eight of 18 strains that did not cause the BBS-type pod reaction failed to cause foliar symptoms or caused symptoms atypical of BBS. However, 10 strains that did not cause the BBS-type pod reaction caused foliar symptoms that were indistinguishable from those caused by strains isolated from BBS lesions (Table 4). Thus, inoculation of bean leaves in the greenhouse did not allow reliable detection of the BBS pathogen.

Table 2. Reference strains of *Pseudomonas syringae* pathovars and *P. viridiflava* used in this study

	Identification number	Species of origin	Source
P. s. pv. *apii*	PJ-1	Celery	R. Dickey, NY
P. s. pv. *aptata*	PA-19	Sugar beet	R. Dickey, NY
P. s. pv. *coronafaciens*	PC-17	Oats	R. Dickey, NY
P. s. pv. *delphinii*	PD-2	Delphinium	R. Dickey, NY
P. s. pv. *glycinea*	PG-4, PG-4TYP, PG-6TE, PG-6TYP	Soybean	R. Dickey, NY
P. s. pv. *lachrymans*	PL-3	Cucumber	R. Dickey, NY
P. s. pv. *maculicola*	83-02, 83-05, 83-14	Cauliflower	R. Campbell, CA
P. s. pv. *morsprunorum*	Psm-5, Psm-6	Cherry	T. Burr, NY
P. s. pv. *papulans*	Psp-5, Psp-9, Psp-17, Psp-21, Psp-29	Apple	T. Burr, NY
P. s. pv. *phaseolicola*	Psph-1, Psph-2, Psph-3, Psph-8	Bean	J. Hunter, NY
	R2A, R2B		D. Hagedorn, WI
	NY1, S&G		D. Webster, ID
	F82-7, F83-18B, F84-4, F84-5, F84-23B, F84-24C, F84-24D, F84-38, WK4-II3A, WK4-IV-3B, WK5-IV2A, WK6-III2A, WK6-12A		H. Schwartz, CO
	P23, MI-1, MI-3		A. Saettler, MI
	Psp-WIS, Psp-Field, Psp-II-5, Psp 83K2, Psp HB38, Psp L42, Psp TOTI 650		J. Venette, ND
	C-267, C-292, C-293		N. Schaad, ID
P. s. pv. *pisi*	103-1-2-1-2	Pea	M. Powelson, OR
	Psps-1, Psps-2, Psps-3, Wis		J. Hunter, NY
P. s. pv. *ribicola*	Unknown	Currant	R. Dickey, NY
P. s. pv. *savastanoi*	PT-10	Oleander	R. Dickey, NY
P. s. pv. *sesami*	PT-22	Sesame	R. Dickey, NY
P. s. pv. *syringae*	Pss-7, Pss-8, Pss-9, Pss-10, Pss-12, Pss-13, Pss-15, Pss-17	Apple	T. Burr, NY
	Pss-14	Sour cherry	T. Burr, NY
	Pss-24, Pss-27, Pss-37	Cherry	T. Burr, NY
	Pss-40, Pss-43	Pear	T. Burr, NY
	Ps-7	Poplar	R. Dickey, NY
	Ps-8, Ps-41, Ps-45	Pear	R. Dickey, NY
	Ps-9	Lilac	R. Dickey, NY
	Ps-10	Lima bean	R. Dickey, NY
	Ps-27	Peach	R. Dickey, NY
	Ps-30, Ps-SD10	Corn	R. Dickey, NY
	Ps-SD7	Sorghum	R. Dickey, NY
	Ps-SD438	Wheat	R. Dickey, NY
	Ps-21	Cherry	R. Dickey, NY
	Ps-33	Sour cherry	R. Dickey, NY
	190-38, 211-50-1, 209-50-2	Pea	M. Powelson, OR
	B-5	Peach	D. Gross, WA
	B-301D, W4N20, W4N22, W4N29, W4N54	Pear	D. Gross, WA
	W4N15, W4N95	Apple	D. Gross, WA
	5D443, 5D447	Cherry	D. Gross, WA
	5D4198	Prune	D. Gross, WA
	5D425	Apricot	D. Gross, WA
	464	Corn	D. Gross, WA
	Ps-1, Ps-10	Peach	D. Ritchie, NC
	B-15+	Almond	J. DeVay, CA
P. s. pv. *tabaci*	PT-3, PT-4, PT-6	Tobacco	R. Dickey, NY
P. s. pv. *tomato*	Pst-1, Pst-2	Tomato	J. Hunter, NY
	PT-7		R. Dickey, NY
P. s. pv. *viburni*	PV-2	Viburnum	R. Dickey, NY
P. viridiflava	PV-1, PV-2, PV-3, PV-4	Parsnip	J. Hunter, NY
	57770-2, 5777-3, 5777-4	Tomato	J. Jones, FL
	5798-3	Pepper	J. Jones, FL

DISCUSSION

Our results support the hypothesis that a unique type of *P. s.* pv. *syringae* is the cause of BBS. Strains of *P. s.* pv. *syringae* isolated from BBS lesions caused a characteristic reaction in inoculated pods, whereas with but one exception all strains of *P. s.* pv. *syringae* from other hosts caused necrotic reactions. Saad and Hagedorn (27) obtained similar results using relatively few strains and primarily leaf assays. They concluded that strains isolated from BBS lesions were more

Table 3. Reactions of excised bean pods (cultivar Bush Blue Lake 274) inoculated with strains of *Pseudomonas* from bacterial brown spot (BBS) lesions on bean and from other sources

Inoculum	Source	Number of strains evaluated	Number of non-BBS-type strains[a]
P. syringae pv. *syringae*	BBS lesions	1,326	10
	Hosts other than bean	45	44
Other *P. syringae* pathovars	16 plant species	68	68
P. syringae	Weed epiphytes	337	314[b]
P. viridiflava	3 plant species	8	8

[a] Strains that did not cause the BBS-type pod reaction.
[b] All 23 BBS-type strains were recovered from weeds adjacent to bean fields with BBS.

Table 4. Results of inoculation of bean leaves in the greenhouse and excised bean pods with strains of *Pseudomonas syringae* pv. *syringae* recovered from bacterial brown spot (BBS) lesions on bean and from other sources

Experiment number	Source	Number of strains	Pod reaction[a]	Leaf reaction[b]	
				BBL 274	BBL 92
1	BBS lesions	2	+	+	ND[c]
	Epiphytes[d]	2	+	+	ND
		3	−	+	ND
		5	−	−	ND
2	BBS lesions	4	+	+	+
	Epiphytes[e]	10	+	+	+
		7	−	+	+
		3	−	−	−

[a] + indicates the BBS-type reaction on excised pods of the cultivar Bush Blue Lake 274.
[b] + indicates BBS symptoms on leaves of the cultivars Bush Blue Lake 274 (BBL 274) and Bush Blue Lake 92 (BBL 92) in greenhouse tests.
[c] Not determined.
[d] Collected from weeds adjacent to beans with BBS.
[e] Collected from bean leaves.

virulent on beans than strains from other sources. The uniqueness of the strains that cause BBS was also noted by Rudolph (25). He proposed that the pathogen be designated *P. s.* pv. *phaseoli* after comparing symptoms in beans inoculated with seven bean strains (four from West Germany and three from the United States) and 13 strains from other hosts.

A few (0.8%) strains of *P. s.* pv. *syringae* isolated from BBS lesions caused a necrotic reaction in inoculated pods. These atypical strains may not have been the primary pathogen in the lesions from which they were isolated, since non-BBS-type *P. s.* pv. *syringae* is often recovered from washes of bean leaves (D. E. Legard, *unpublished data*).

Our results with over 1,300 strains isolated from BBS lesions confirm the usefulness of the pod assay to identify strains capable of causing BBS. However, the reliability of the bean pod assay depends upon the use of greenhouse-grown pods in the early stage of seed formation, the use of 24- to 48-hr-old bacterial cultures, and scoring of the results over a 4-day period. The cultivar is also important, because differences in susceptibility have been noted in the field (15), and this difference was apparent when pods of different cultivars were used in the bioassay (J. E. Hunter, *unpublished data*). A bean with determinant-type flowering should be used to ensure a plentiful supply of pods with minimal greenhouse space. Also, to obtain reliable results the scorer must carefully examine lesions for necrotic reactions indicative of strains of *P. s.* pv. *syringae* that do not cause BBS.

A major advantage of our pod inoculation procedure is that it eliminates the need for cell suspensions of a known concentration. Thus, large numbers of strains were quickly evaluated for their ability to cause BBS. Our inability to recover the BBS pathogen from weeds outside of bean-growing areas, and only rarely from weeds near bean fields with a recent history of the disease, suggests that this type of *P. s.* pv. *syringae* does not occur as a common epiphyte on many species of weeds in New York State. Also, the uniqueness of strains isolated from BBS lesions helps to explain why this disease has not previously been a problem on beans in New York State even though the crop has been grown for many years near fruit crops with a history of diseases caused by *P. s.* pv. *syringae*. The BBS pathogen may have been introduced only recently on bean seed, since it is known to be seedborne (14). Further work needs to be done to determine if the pathogen has an epidemiologically important epiphytic phase on plant species other than bean in New York State, as has been reported in Wisconsin (10,20).

Assays conducted on leaves of plants in the greenhouse required more time and space than the pod assay, symptoms were not always distinct or consistent, and several plants had to be used, because some escaped infection or few lesions developed. Strains of *P. s.* pv. *syringae* that cause the BBS-type pod reaction consistently caused BBS symptoms in greenhouse tests. Because several strains that do not cause the BBS-type pod reaction incited foliar symptoms similar to those caused by BBS strains, important questions are raised about the degree of relatedness of strains from various sources as well as the suitability of this greenhouse leaf test for accurately identifying strains of *P. s.* pv. *syringae* associated with BBS in the field. Therefore, we conclude that the pod assay is easier and more reliable than whole-plant leaf assays.

Because standard microbiological methods do not differentiate between strains of *P. s.* pv. *syringae* pathogenic to bean and strains pathogenic to other hosts, we consider the pod assay to be an essential procedure in etiological or epidemiological studies of BBS. The pod assay also can be used reliably for similar studies involving *P. s.* pv. *phaseolicola*, the causal agent of halo blight of bean. Because of the uniqueness of the symptoms induced by both these bean pathogens, confusion should not arise if *P. viridiflava* or other pathovars of *P. syringae* are encountered in studies using the pod assay.

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