New Assays for Detection of *Pseudomonas syringae* pv. *glycinea* in Soybean Seed

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ABSTRACT

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Two new assays for seedborne *Pseudomonas syringae* pv. *glycinea,* the causal agent of bacterial blight in soybeans, were compared with two existing assays on 21 commercial soybean seed lots. For one of the new assays (C), seeds were ground, suspended in saline, and plated on King's B medium amended with 10 μg/ml cephalexin (KBC). Presumptive colonies were confirmed as *P. s. glycinea* by pathogenicity to soybean seedlings and by agglutination with antiserum. For the other new assay (D), seeds were washed under running tap water, dried and plated on KBC, and presumptive colonies confirmed as *P. s. glycinea* in the same way as in assay C. For one of the existing assays (A), seeds were soaked in sterile water, the soak solution plated on KBC, and presumptive colonies of *P. s. glycinea* confirmed by a positive reaction in the levan test and negative reactions in oxidase and esculin hydrolysis tests. For the other existing assay (B), seeds were soaked in a buffer solution, the soak solution centrifuged, the resuspended pellet plated on KBC, and presumptive colonies confirmed as *P. s. glycinea* by immunofluorescence. Assay C consistently detected higher numbers of colony-forming units of the pathogen than did assays A and B and was completed in 7 days compared with 12 and 15 days for B and A, respectively. Although a different unit of measurement (percentage of infected seeds) and a tenfold smaller number of seeds was used in assay D, results suggested that the assay had a high degree of sensitivity. Rankings for incidence of seedborne *P. s. glycinea* across the 21 seed lots were strongly correlated ($r = 0.50 - 0.97$) between all assays. The validity of the nutritional, serological, and pathological tests that were components of the different assays was confirmed when all of the 121 strains of *P. s. glycinea* obtained in the four assays gave the correct reaction when submitted to each test.

Bacterial blight, caused by *Pseudomonas syringae* pv. *glycinea* (Coerper) Young, Dye and Wilkie, is prevalent on soybeans (*Glycine max* (L.) Merr.) in cool temperate regions of the world (4,5,7,16). The pathogen is seedborne, with incidences of seed infection levels greater than 90% reported (6,9,11), and it can be transmitted by seed to the subsequent soybean crop (11). Although yield has been reduced by up to 17.9% in plants artificially inoculated under field conditions (12,17), the disease has not been shown to cause significant yield losses under conditions of natural infection. The disease is an economic problem for soybean seed producers in the U.S., however, because phytosanitary regulations require that seed lots exported to the European Community (EC) be tested for the pathogen.

Several methods have been reported for detection of *P. s. glycinea* in soybean seed lots, including direct plating on media (9), grow-outs of seedlings (10,11), serological testing (1), and inoculation of plants with seed extracts (3), but the one used for phytosanitary certification is an unpublished protocol developed in France (J.-F. Chauveau, *personal communication*) and mutually agreed upon in 1989 by U.S. and EC authorities. Neither this method nor the others have been standardized in international testing programs. Furthermore, because of the time and labor involved, all of these assays are expensive to run and create logistical problems when large numbers of samples have to be tested over short periods. The objective of this study was to develop an assay for *P. s. glycinea* in soybean seed that would be at least as sensitive as existing protocols and would reduce time and labor.

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MATERIALS AND METHODS

Assays. Twenty-one commercial soybean seed lots of different cultivars, previously assayed for seedborne infection by *P. s. glycinea* by the Seed Health Testing Service at Iowa State University using the protocol developed by J.-F. Chauveau (*unpublished*), were selected to represent a wide range of natural incidence of seed infection. All 21 seed lots were tested with Chauveau's assay (A), an assay (B) developed in Italy (1), and two new assays (C and D) developed in our laboratory. For assays A, B, and C, five replicates of 100 g of seeds (approximately 1,000 seeds) were tested for each seed lot. For assay D, five replicates of 100 seeds were tested for each seed lot. Bacteria used as positive and negative controls in each assay included *P. s. glycinea* and *Xanthomonas campestris* pv. *campestris* (Smith) Dye both from our laboratory, and *Pseudomonas aeruginosa* (Shroeter) Migula, supplied by B. W. Kennedy (University of Minnesota).

In assay A, seeds were soaked for 24 hr at 4–5 C in 600 ml of sterile tap water adjusted to pH 6.5 with a phosphate buffer solution. Threefold serial dilutions were made from the soaking solution and 0.1-ml aliquots plated on King's B medium (13) amended with 10 mg/ml cephalexin (KBC) (8). After incubation at 25 C for 2–3 days, presumptive colonies of *P. s. glycinea*, exhibiting a blue fluorescence under UV light (370 nm), were reisolated onto KBC and confirmed as *P. s. glycinea* by a positive reaction for levan production and negative reactions in oxidase and esculin hydrolysis tests (13).

In assay B, seeds were soaked in 600 ml of sterile buffer (pH 7.2) for 24 hr at 4–5 C. A 40-ml portion of the soaking solution was then centrifuged for 20 min at 12,800 rpm, and the pellet resuspended in 1 ml of sterile buffer. Presumptive colonies of *P. s. glycinea* were obtained as in assay A and confirmed as *P. s. glycinea* by immunofluorescence as described by Calzolari (1). The polyclonal antiserum was produced in rabbits against *P. s. glycinea* (509 OMP/Bo) by A. Calzolari, Osservatorio Regionale per le Malattie delle Plante, Bologna, Italy.

For assay C, dry seeds were ground in a Stein Mill for 1 min, then added to 600 ml of sterile saline (0.85% NaCl) and the mixture placed on a rotary shaker for 2 hr at 25 C at 220 rpm. Presumptive colonies of *P. s. glycinea* were obtained as in assay A and confirmed as *P. s. glycinea* by pathogenicity and slide agglutination tests. Pathogenicity was determined by inoculating 15-day-old, greenhouse-grown soybean seedlings (cvs. Oakland, Beeson, Acme, and Flambeau) by rubbing leaves with a sterile cotton swab dipped in an aqueous suspension of the strain (approximately 10^5 cfu/ml). The seedlings were incubated in light for 48 hr at 90% relative humidity in a mist chamber at 25 C, then transferred to the greenhouse

Table 1. Responses of *Pseudomonas syringae* pv. *glycinea* and other bacteria isolated from soybean seeds to pathological, biochemical, and serological tests

Isolates						Biochemical[d]			
Bacterium	**Source**	**No.**	**Path.[a]**	**Fluor.[b]**	**Ser.[c]**	**Ox.**	**Lev.**	**Esc.**	**Pro.**
Pseudomonas syringae pv. *glycinea*	Seeds[e]	121	+	+	+	−	+	−	−
	ISU[f]	2	+	+	+	−	+	−	−
	UM[g]	1	+	+	+	−	+	−	−
	France[h]	6	+	+	+	−	+	−	−
P. syringae pv. *glycinea*, Race 2	UM[g]	6	+	+	−	−	+	−	−
P. fluorescens	Seeds[e]	129	−	+	−	+	+	−	+
P. putida	Seeds[e]	2	−	+	−	+	−	+	−
Pseudomonas spp.	Seeds[e]	8	−	−	−	−	+	+	+
Erwinia herbicola	Seeds[e]	48	−	−	−	−	+	+	+

[a] Pathogenicity on soybean seedlings.
[b] Fluorescence on King's B medium.
[c] Slide agglutination serological test.
[d] Biochemical tests include oxidase (Ox.), Levan (Lev.), esculin hydrolysis (Esc.), and protease (Pro.).
[e] Isolates from soybean seeds from the four assays for *Pseudomonas syringae* pv. *glycinea* described in this study.
[f] Cultures from E. J. Braun, Iowa State University.
[g] Cultures from B. W. Kennedy, University of Minnesota.
[h] Cultures from J.-F. Chauveau, France.

and observed for necrotic lesions on leaves 4–7 days after inoculation. For the slide agglutination test, 10 μl of bacterial suspension in water (10^5 cfu/ml) were mixed in polystyrene Micro ELISA plates (Dynatech Corp.) with 10 ml of a 1:1,000 aqueous dilution of the antiserum obtained from A. Calzolari. The plates were agitated for 1 hr at 25 C on a rotary shaker at 220 rpm, and agglutination was determined under a stereoscopic microscope.

For assay D, seeds were washed under running tap water for 1 hr, air dried on the laboratory bench for 10 min, plated on KBC at 20 seeds/plate, and incubated at 25 C for 2–3 days. Isolations were made onto KBC from blue fluorescent bacterial colonies that formed around seeds. These were confirmed as *P. s. glycinea* by pathogenicity and slide agglutination tests as used in assay C.

Characterization of bacterial strains on seeds. A group of strains, representative of the seedborne bacterial microflora found in the above assays, was selected at random from the various assays. They were identified according to Schaad (13) and included: *P. s. glycinea; P. fluorescens* (Trevison) Migula and *P. putida* (Trevison) Migula, both with green fluorescence; nonfluorescent *Pseudomonas* spp.; and *Erwinia herbicola* (Lohnis) Dye strains with nonfluorescent white and yellow pigments. Strains were lyophylized in equal volumes of 5% peptone and 20% sucrose and stored at 4 C before testing. These strains and a group of *P. s. glycinea* strains from other sources (Table 1) were used to compare the reactions of the various diagnostic tests that were components of the four assays. Also included in the experiment were several positive and negative control strains including *Pseudomonas syringae* pv. *syringae* van Hall, *P. syringae* pv. *phaseolicola* (Burkholder) Young et al, *P. cepacia* Burkholder, *Erwinia carotova* subsp. *carotovora* van Hall, and *X. campestris* pv. *campestris*. Cultures were grown for 24 h at 27 C on Difco Nutrient agar (NA), then were tested for fluorescent pigment production on KBC, gram stain (15), and reactions in oxidase, protease (gelatin liquefaction), levan, esculin hydrolysis, pathogenicity and slide agglutination tests. Tests were repeated twice for each strain.

Table 2. Comparison of four assays for *Pseudomonas syringae* pv. *glycinea* in soybean seed lots

	Assay[a]			
Seed lot	**A[b]**	**B[c]**	**C[d]**	**D[e]**
1	57	0	3,400	58.5
2	225	150	2,150	37.0
3	129	100	2,000	25.2
4	34	0	1,600	17.7
5	4	0	1,300	12.7
6	24	0	900	6.5
7	0	0	100	4.0
8	0	0	90	3.8
9	0	0	50	2.5
10	2	25	40	1.2
11	0	10	18	0.7
12	6	3	10	0.2
13	5	0	9	0.3
14	0	6	5	0.2
15	2	0	5	0.1
16	0	5	4	0.1
17	0	0	2	0.0
18	0	0	0	0.0
19	0	0	0	0.0
20	0	0	0	0.0
21	0	0	0	0.0
Means	23.2	14.2	556.0	8.1

[a] Values for assays A, B, and C are expressed as colony-forming units/gram of seeds for five replicates of 1,000 seeds; for assay D, as percentage of infected seeds for five replicates of 100 seeds.
[b] Seeds soaked in sterile water; soak solution plated on King's B medium amended with 10 μg/ml cephalexin (KBC); presumptive colonies of *P. s. glycinea* confirmed by negative reactions in oxidase, levan, and esculin hydrolysis tests.
[c] Seeds soaked in buffer; soak solution centrifuged; the pellet resuspended and plated on KBC; presumptive colonies confirmed as *P. s. glycinea* by immunofluorescence.
[d] Ground seeds suspended in saline; plated on KBC; presumptive colonies confirmed as *P. s. glycinea* by pathogenicity to soybean seedlings and slide agglutination.
[e] Seeds were washed under running tap water; dried and plated on KBC; presumptive colonies were confirmed as *P. s. glycinea* by pathogenicity and slide agglutination tests.

RESULTS

Significantly higher numbers of colony-forming units of *P. s. glycinea* were detected from seed extracts by assay C than by assays A and B as determined by comparison of mean values, by Student's *t* test (Table 2). Assay D could not be directly compared with the other assays because it had a different unit of measurement, but the high percentage of

infected seeds detected in some seed lots suggested that the method was sensitive. Rankings for incidence of seedborne *P. s. glycinea* across the 21 seed lots were strongly correlated among all assays, with a particularly high correlation coefficient found between the two new assays, C and D (Table 3).

A total of 121 bacterial strains were identified as *P. s. glycinea* in the four assays. Each of these strains gave the correct reaction for *P. s. glycinea* when submitted to each of the diagnostic tests that were components of the four assays (Table 1). This also was true for nine *P. s. glycinea* strains obtained from other sources. The diagnostic tests differentiated other bacteria isolated from the seed extracts from *P. s. glycinea* as follows: strains of *P. fluorescens* reacted negatively in antiserum and pathogenicity tests and positively on oxidase and protease tests; *P. putida* reacted negatively on the levan, antiserum, and pathogenicity tests and positively on the esculin test; *E. herbicola* reacted positively on the esculin and protease tests and negatively on the slide agglutination and pathogenicity tests. Although all diagnostic tests clearly differentiated *P. s. glycinea* from other common seedborne bacteria of soybeans, there was evidence for differences within the *P. s. glycinea* strains tested. On KBC medium, strains of *P. s. glycinea* produced two distinct colony types, one translucent with an entire margin and raised surface, (90% of the strains) and the other (approximately 10% of the strains) with an indented margin, rough surface, and light brown color. All strains of *P. s. glycinea* expressed well-defined symptoms of pathogenicity, whereby water-soaked lesions turned yellow to brown; the centers then dried out, turned black, and were surrounded by a water-soaked margin bordered by a yellowish halo, but differences in severity of symptom development were evident between strains. Cross and Kennedy (2) also reported variability in pathogenicity among strains of *P. s. glycinea*.

Table 3. Pearson correlation coefficients between results of three assays for *Pseudomonas syringae* pv. *glycinea* in soybean seed lots

	A		B		C	
Assay	***r***	***p***	***r***	***p***	***r***	***p***
B	0.96	0.0001				
C	0.68	0.0006	0.50	0.02		
D	0.70	0.0004	0.51	0.02	0.97	0.0001

DISCUSSION

The new assay C clearly had greater sensitivity for detection of *P. s. glycinea* in soybean seeds than did the existing assays, A and B. Assay C also was completed in approximately half the time needed for the existing assays. Time was saved in assay C because nutritional tests were not required after isolation onto KBC. Although no quantitative measurements were made, it was obvious that fewer contaminants developed during the isolation phase on KBC in assay C than in assays A and B. This is likely a major reason for the improved sensitivity of assay C. Reduced contamination also minimized the need to subculture presumptive colonies of *P. s. glycinea* from KBC for subsequent confirmatory tests, a time- and labor-consuming aspect of assay A. The reduced degree of contamination in assay C is in line with Schaad's (14) statement that contamination is likely to be lower when seeds are ground and plated directly onto media, than when they are exposed to long soak periods before plating as in assays A and B.

The high degree of sensitivity of assay D also seemed to be related to reduced contamination of plates as evidenced in a preliminary test in which pre-washing the seeds under tap water for 1 hr before plating them on KBC greatly reduced growth of saprophytic microorganisms, and enhanced growth of *P. s. glycinea*. Assay D also was completed in 9 days, compared with 12 and 15 days for assays B and A, respectively. This assay is unlikely to be acceptable as an alternative method, however, because only 100 seeds per replicate were tested. Unacceptable logistical problems probably would develop by increasing replicate size to the 1,000 seeds used in the other assays.

The consistency of reactions in the diagnostic tests used in the four assays indicated that each assay could accurately detect *P. s. glycinea* in seeds. Also, the most commonly found bacteria in soybean seeds were easily differentiated from *P. s. glycinea* by the range of reactions to the tests used in each assay. The antiserum developed by Calzolari in Italy was highly specific for strains of *P. s. glycinea* from the United States and proved to be an effective diagnostic tool.

Present phytosanitary regulations agreed upon by U.S. and EC authorities require that the pathogen must be detected by assay A in each of five subsamples of 1,000 seeds for each seed lot, before a seed lot is denied a phytosanitary certificate. Although assay C saves time and labor, it should not replace assay A until further research is done to determine tolerance levels to compensate for its greater sensitivity.

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