Ecology and Epidemiology

A New Race of *Pseudomonas glycinea*

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ABSTRACT

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The pathogenic response of seven soybean cultivars to a previously undescribed race of *Pseudomonas glycinea* is reported. For the purpose of this study, we defined the pathogenic response in terms of resistance and susceptibility. Similarities to selected characteristics of the seven previously described races, specifically pigment production and xylose utilization, were studied. The value of relying on nutritional tests for bacterial species differentiation is considered, since mutants of the new race were isolated which mimic some of the other races. A convention for the use of biochemical tests is proposed.

Cross et al (3) reported the existence of pathogenic races of *Pseudomonas glycinea*. They defined seven races of *P. glycinea* by resistant/susceptible reactions of differential soybean cultivars. This system of distinct pathogenic responses could provide a model for the mechanism of pathogenicity provided that the specific genes controlling the various reactions can be identified. First, however, a greater understanding of similarities among the races is needed.

If the taxonomy of the genus *Pseudomonas* were less confused, the genetic similarity of the different races might be clearer. Several authors have reported attempts to separate the *Pseudomonas* spp. by numerous biochemical and nutritional tests (9,10,13,14,17).

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dematium var. *truncata*, and *C. gloeosporioides* was common in field plants (Table 3). None of these fungi was isolated from more than 1% of the velvetleaf seeds that were assayed. No infection by these fungi was found in the earliest-emerging group of velvetleaf seedlings, but on later-emerging plants, these fungi always were detected on 2-wk-old plants. *C. dematium* var. *truncata* colonized the vast majority of stem sections early, but the incidence appeared to decline as the season progressed. *P. sojae* infected 25–35% of stem sections throughout the season and the incidence exceeded 50% at the season's end. The incidence of *C. gloeosporioides* was low during most of the season, but rose noticeably at the end. Rates of recovery of these three fungi were similar for both soybean and velvetleaf from the same field. Stem infection by these fungi never exceeded 10% on velvetleaf plants growing in an abandoned field never cropped to corn or soybeans. In survey of 10 soybean fields scattered througout Champaign County *P. sojae* was isolated from 35 to 65% of plants collected in mid-September (Table 4).

TABLE 4. Percentage *Phomopsis sojae* recovery from the seventh stem internode of velvetleaf (*Abutilon theophrasti*) at 10 soybean field sites surveyed in Champaign County, IL, in September 1978

Site number[a]	Plants (no.)	*P. sojae* isolated (%)
1	15	40
2	24	50
3	20	60
4	20	65
5	15	27
6	15	33
7	20	35
8	20	30
9	16	62
10	20	40
		$\overline{X} = 44.9$

[a]To survey the county, 10 equal-sized areas were designated; within 2 km from the center of each area, a soybean field sampling site was chosen and 15 to 20 velvetleaf plants were selected at random from each site.

All isolates used in pathogenicity tests on soybean pods originated from latent infection of soybean and velvetleaf stems. Pathogenicity of *P. sojae* isolated from soybean overlapped that of velvetleaf isolates (Table 5). Generally, isolates from velvetleaf were more pathogenic on soybean pods than were those from soybean. No cultural differences were noted regardless of host origin. All isolates produced concentric rings of stromatic tissue on PDA. Soybean and velvetleaf isolates of *C. dematium* var. *truncata* were similarly pathogenic to pods. *C. gloeosporioides* was not pathogenic to soybean pods. Both of these *Colletotrichum* spp. reduced the seedborne incidence of *P. sojae* (Table 5).

Depending on host origin, growth differences were found for both *P. sojae* and *C. dematium* var. *truncata* isolates on SEA. Soybean isolates of each fungus grew more slowly on SEA than did velvetleaf isolates. An isolate of *P. sojae* from soybean, which would not grow on SEA, was avirulent on soybean pods. Differences of growth of isolates based on their host origin were not evident on VEA.

DISCUSSION

This is the first report of velvetleaf stem canker. Kmetz et al (8) found that nonstromatic isolates of *P. sojae* from soybean produced stem cankers and were less pathogenic on soybean seeds than were stromatic isolates. Similar results were obtained with stem canker isolates from velvetleaf.

In the past, speciation of *Phomopsis* and *Colletotrichum* was based largely on host. Two morphologically distinct *Colleto-*

TABLE 5. The pathogenicity of *Colletotrichum dematium*, *Colletotrichum gloeosporioides*, and *Phomopsis sojae* from soybean (*Glycine max*) and velvetleaf (*Abutilon therophrasti*) on soybean pods and seeds[a] and the growth of these fungi on soybean extract agar (SEA) and velvetleaf extract agar (VEA)[b]

Fungus	Origin	Isolate no.	Germ-ination[c]	Pod lesions[c]	Infected pods[c]	Seedborne *Phomopsis sojae*[c]	Seedborne *Colletotrichum* species (%)	Growth[d] SEA	Growth[d] VEA
P. sojae	Soybean	3	108[d]	96	95	92	0	0.0	32.0
		5	50	160	150	160	0	8.0	36.7
		6	22	323	226	323	0	5.3	36.5
	Velvetleaf	10	78	251	166	272	0	13.7	39.8
		11	31	300	214	313	0	16.7	34.5
		12	67	236	178	236	0	9.2	34.8
C. dematium	Soybean	2	136	204	179	44	34	3.3	20.8
		3	167	68	95	24	18	4.2	21.3
		5	140	80	107	68	11	7.0	20.8
	Velvetleaf	10	97	308	213	47	72	10.5	19.8
		11	143	317	214	72	64	9.8	17.0
		12	131	188	154	72	32	9.3	18.8
C. gloeosporioides	Soybean	1	90	64	72	60	4	...[f]	...
		2	110	118	107	62	5	...	...
	Velvetleaf	11	105	78	72	14	18	...	...
		12	113	146	142	72	3	...	...
FLSD[g] ($P = 0.05$)			28	60	23	40	15	1.5	1.2

[a]Surface-disinfested Amsoy 71 soybean pods were used at green full-bean stage for inoculation. To prepare inoculum, 15 ml of water was added to 9-cm-diameter culture plates containing a 2-wk-old culture of the test fungus on potato dextrose agar. Surface mycelium was scraped free and the mix of mycelium and water was ground with mortar and pestle. Three drops of inoculum were applied by medicine dropper for every pod tested.

[b]For preparing media 4-wk-old velvetleaf stems or soybean pods with full-sized green beans were used. Materials were homogenized and filtered. Three hundred milliliters of each filtrate were added to 700 ml of water containing 15 g of agar.

[c]Values expressed as percentage of noninoculated control having 38% germination, 25% pod lesions, 42% infected pods, and 25% seed infection by *P. sojae*.

[d]Increase of colony diameter (millimeters) growing from a 5-mm-diameter plug for 3 days at 25 C on soybean or velvetleaf extract agars.

[e]Means are based on two replications each of 10 soybean pods.

[f]No data.

[g]Fischer's least significant difference, $P = 0.05$.

This has been successful in reducing the number of *Pseudomonas* species first described by host range (7,15). However, when these tests are related to *P. glycinea*, there are many inconsistencies among different isolates. For example, Sands et al (14) reported testing three races of *P. glycinea* and, by establishing similarity coefficients, placed each of them in different subgroups of the fluorescent phytopathogen group I. Also, Misaghi and Grogan (10) reported that none of four *P. glycinea* isolates utilized L(+)arabinose while all could utilize D-gluconate as the sole carbon source (10); in contrast, Sands et al (14) reported that at least one of their *P. glycinea* isolates could utilize L(+)arabinose, but not D-gluconate.

More recently, other methods have been used to differentiate *Pseudomonas* spp. Flagellar characteristics (16), DNA base ratios (2,8), phage sensitivity and serology (1,11), and DNA homology (12) all have been used to classify phytopathogenic pseudomonads into various subgroups. However, due to the lack of distinct characteristics and to variability of results, most of the arginine dihydrolase-negative, hypersensitive-positive, green-fluorescent pseudomonads (including *P. glycinea*) have been designated as pathovars of *P. syringae* (5,18). A study of bacterial proteins by gel electrophoresis may help to determine whether these pathovars belong in a single species (4).

The ability to utilize xylose as the sole carbon source has been reported as useful for distinguishing members of the subgroups of phytopathogenic pseudomonads (14). Also, it has been observed that pathogenic *P. glycinea* race 2 isolates produce a characteristic browning of beef-extract-peptone and complete media (3). This would appear to be a distinct marker for race 2. The use of such characteristics as the ability or inability to utilize a particular carbon source or the production of a pigment could be misleading. It is possible that all races of *P. glycinea* have the necessary genetic information to utilize various carbon sources and produce different pigments, but that these genes are not expressed.

We have used the pathogenic response on indicator soybean cultivars to define a previously unreported race of *P. glycinea*. We then attempted to use characteristics other than pathogenicity and host range for race determination. The results of these studies are reported here.

MATERIALS AND METHODS

Strains. Isolates of *P. glycinea* races 1 through 7 were obtained from B. W. Kennedy, University of Minnesota, through the courtesy of N. T. Keen, University of California, Riverside. The isolate of the new race, here designated as PgB3, originally was isolated in 1970 from an unknown soybean cultivar by M. L. Moffett, Brisbane, Australia. PgB3 *met-25 nalA2* and PgB3 *met-25 his-51 nalA2* are virulent, ethyl methane sulfonate (EMS)-induced mutants of PgB3 which require methionine or methionine and histidine. The mutants also are resistant to 2 mg/ml nalidixic acid.

Media. Complete medium (1% casein hydrolysate, 0.5% yeast extract, and 0.4% K_2HPO_4) and minimal medium [0.7% K_2HPO_4, 0.15% KH_2PO_4, 0.1% NH_3NO_3, 0.01% $Mg(SO_4)_2$] were autoclaved separately from Difco Bacto Agar (1.5%). Appropriate supplements were added after autoclaving at the following final concentrations: glucose, 1%; xylose, 0.2% or 1%; methionine, 20 μg/ml; histidine, 20 μg/ml; and nalidixic acid, 1 mg/ml. Both media have an approximate pH of 7.4 which allows solubility of nalidixic acid when added from a 100 mg/ml stock solution, pH 13.

Mutagenesis. Log phase cells (10^8–10^9 colony-forming units [cfu]/ml) were treated with 0.5% EMS for 4 hr at 25 C, then either diluted to form single colonies and plated on complete agar to screen them for pigment production or plated directly on minimal agar supplemented with xylose instead of glucose.

Pathogenicity test. In all cases, inoculum for pathogenicity tests was single-colony clones streaked from subcultures which were stored either in saline at 4 C or in 25% glycerol at −15 C. The original cultures are stored in lyophil. Unifoliate leaves of 10- to 20-day-old soybean were inoculated with a Hagborg device (6). Both low levels (1×10^6 cfu/ml) and high levels (up to 1×10^8 cfu/ml) of inoculum were compared. Soybean seed was obtained from B. W. Kennedy, University of Minnesota.

Xylose utilization. Single colonies were streaked on minimal agar supplemented with either glucose or xylose. Alternately, 0.1 ml of an overnight culture (10^8–10^9 cfu/ml) was spread on plates containing either medium.

Pigment isolation. Broth shake cultures were grown for 24–48 hr, centrifuged, and the supernatants were evaporated to dryness. They were then suspended in chloroform, centrifuged, resuspended in ethanol, centrifuged, and the pellets were dried under vacuum. Finally, the pellets were redissolved in sterile H_2O. The carrier solvent for paper chromatography was butanol-saturated H_2O with 20% acetic acid.

RESULTS

In the *P. glycinea*/soybean system, the resistant or incompatible response is characterized by necrosis of the leaf tissue at the inoculation point, usually within 24 hr. The susceptible or compatible response has little necrosis after 5 days, but does have watersoaking with or without chlorosis spreading from the inoculation point. The intermediate reaction is characterized by more necrosis and less watersoaking and chlorosis than the compatible reaction. These reactions were photographically defined by Cross et al (3). Single colony clones of PgB3 constantly induce a hypersensitive resistant response with no water-soaking on Lindarin and an intermediate response on each of these other soybean cultivars: Acme, Flambeau, Harosoy, Merit, Norchief, and Chippewa. Isolates of races 1 through 7 tested at the same time generally gave their reported responses (3). Environmental factors can cause variability in the pathogenic response, and we observed variability between tests. However, races 1 through 7 were unambiguously defined by comparing all cultivars in each test. Since none of the previously described races give a similar response, we consider PgB3 to represent a new race, race 8. The extent of its natural distribution is not known, however, we have tested five other isolates of race 8; PgB1, PgB2, PgB4, PgB5, and PgB6. These were isolated between 1970 and 1973 from Kingaroy, Redland Bay, Aratula, and Biloela, all in Australia.

Two pigment producing mutants of PgB3, designated *brn*, were isolated at a frequency of about 2×10^{-6} among the survivors after treatment with EMS. *P. glycinea* race 2 (PgR2), PgB3 *met-25 nalA2 brn-1*, and PgB3 *met-25 his-51 nalA2 brn-2* each produce a browning of complete broth or agar after 24–48 hr of growth. These brown, water-soluble pigments are insoluble in all common organic solvents. The pigments produced by PgR2 and the PgB3 *brn* mutants in chloroform- and ethanol-extracted culture filtrates are indistinguishable by UV and visible spectrophotometry and paper chromatography. PgB3 *met-25 nalA2 brn-1* and PgB3 *met-25 his-51 nalA2 brn-2* give the same response in the plant as wild-type PgB3.

Table 1 show the utilization of xylose by races 1 through 7 (PgR1–PgR7) and PgB3. Spontaneous xyloze-utilizing mutants of PgR1, PgR2, and PgR6 were obtained. More than 50 single-colony isolates representing mutants of all three races were tested for responses in the plant. In all cases these retained the responses of the race from which they were derived. EMS-induced, xylose-utilizing mutants (*xyl*) of PgB3 were isolated. These mutants grew equally well with xylose or glucose as the sole carbon source and gave the same pathogenic response as PgB3. All wild-type *P. glycinea* isolates grew on media containing both xylose and glucose together showing that xylose is not inhibitory in the presence of glucose. EMS-induced xylose-utilizing mutants of multiple auxotrophic strains also were isolated (eg, PgB3 *met-25 brn-1 his-67 xyl-20*); their existence shows that nutrients required by the auxotroph do not serve as a carbon source at the levels supplied.

DISCUSSION

None of the known genes in soybean conferring resistance to *P. glycinea* are effective against PgB3. The molecular basis for the resistant response of Lindarin is not known. On the susceptible cultivars, PgB3 causes an initial necrotic reaction similar to, but

less extensive than, the hypersensitive resistant response. However, after 4 days, watersoaking and chlorosis are present, showing that the resistance is not complete.

In trying to alter differences among the races of *P. glycinea* through mutagenesis, we have not demonstrated a change in the virulence/avirulence response of any isolate that would enable it to mimic another race. If this were possible, the information would be useful in determining the dominance of virulence or avirulence phenotypes and would also be helpful in isolating the specific factors involved in the various responses. Other than pathogenicity, the only distinguishing traits found among the races are xylose utilization and pigment production. However, we have been able to change these easily selectable traits, which may indicate that the races are closely related. Possibly, there exists a series of single-gene differences between the races. The pigment produced by PgR2 and PgB3 "brown" mutants is a useful identifying characteristic, which could have arisen from similar blocks in the same nonessential metabolic pathway that allows a build-up of a pigmented intermediate. Xylose utilization apparently provides little selective advantage to *P. glycinea* and consequently is easily lost. Since mutants for xylose utilization can be isolated, it is probable that the genes for making the necessary enzymes are present but are in an inactive or altered form. We conclude that if enough mutagenized isolates were screened for changes in their virulence/avirulence response, we would be able to detect changes in the pathogenic response that ultimately would provide a description of the mechanism of pathogenicity.

Pseudomonas is a diverse bacterial genus, and many biochemical tests have been proposed to delimit its species. But, it is clear that relying on nutritional tests for the differentiation of *Pseudomonas* spp. may lead to erroneous or simplistic conclusions. We suggest that in making nutritional utilization studies, the possibility of spontaneous or inducible mutation be considered in the event that strain differences are found. If a mutation in a particular strain can be induced or selected, then by convention, that strain would be scored positive for that test. Further, it is important to determine if the mutation is for loss of sensitivity rather than utilization. If xylose utilization were used as a criterion to separate *Pseudomonas* spp., our isolates of races 3, 5, and 7 would be segregated from the others. However, by testing the potential for xylose utilization, we have shown that all of the isolates have the capacity to utilize xylose. It is necessary, when testing nutritional and biochemical differences, to realize that the appropriate genes may be present but not constitutively expressed or inducible for one reason or another.

TABLE 1. Xylose utilization by wild type isolates of *Pseudomonas glycinea*.

	Growth on[a]			Frequency of spontaneous mutation to xylose utilization
	1% Glucose	0.2% Xylose	1% Xylose	
R1	+[b]	−	−	8×10^{-8}
R2	+	−	−	1×10^{-6}
R3	+	+	+	...
R4	+	−	−	$<1 \times 10^{-9}$
R5	+	+	+	...
R6	+	−	−	5×10^{-8}
R7	+	+	+	...
B3	+	−	−	$<1 \times 10^{-9}$

[a] Bacteria were streaked onto minimal agar supplemented with either glucose or xylose and incubated for 2 wk at 25 C. Spontaneous mutation to xylose utilization frequencies were calculated from plates inoculated with 1×10^8 bacteria.
[b] + = growth; − = no growth.

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