Reaction of Cowpea Seedlings to Phytopathogenic Bacteria

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ABSTRACT

Inoculation of cowpea (*Vigna sinensis*) was one of several tests found valuable for rapid identification of isolates of *Pseudomonas syringae*. A total of 413 bacterial cultures were tested, which included 69 isolates of four *Agrobacterium* spp., 23 of seven *Corynebacterium* spp., 55 of 10 *Erwinia* spp., 214 of 35 *Pseudomonas* spp., and 52 of 12 *Xanthomonas* spp. Positive results consisted of formation of brown necrotic lesions on leaves 24 hr after inoculation, when inoculated plants were maintained in a moist chamber. The cowpea variety, 'California Blackeye 3', reacted to two isolates of *A. tumefaciens*, eight of *E. amylovora*, two of *P. eriobotryae*, one of *P. cattleyae*, and all tested isolates of *P. alliicola, P. cichorii, P. pisi, P. syringae*, and *X. vesicatoria*. When 10 cowpea varieties were inoculated with 45 isolates of *P. syringae*, only California Blackeye 3 gave a positive reaction for all isolates.

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Since both saprophytic and phytopathogenic pseudomonads usually can be cultured from the infected tissues of diseased plants, further testing is required to distinguish the saprophytic and pathogenic isolates. Several such tests have been reported. The oxidase (13, 17), arginine dihydrolase (17), and tobacco hypersensitive reactions (6, 8) have been used to differentiate saprophytic pseudomonads from most phytopathogenic bacteria prior to testing them for pathogenicity. Considerable reliance has been placed on nutritional requirements as an aid to grouping phytopathogenic pseudomonads (11, 13, 17). De Ley (2) reported that DNA base composition serves as an important criterion for determining relationships among phytopathogenic bacteria. Phage typing (4) and serology (12, 14) have been used for differentiating pathogenic pseudomonads and for separating them into subgroups at the intraspecific level. Gel electrophoresis of soluble proteins has been increasingly utilized as a taxonomic tool in the study of microorganisms, with particular application to plant pathogens (7, 15). However, utilization of an indicator plant as a supplementary aid in identifying a phytopathogenic pseudomonad(s) has not been reported.

Cowpea (*Vigna sinensis*) has been widely used in the identification of viruses (16, 18, 19, 21). Therefore, we tested the reaction of cowpea seedlings to our collection of different phytopathogenic bacteria, with particular emphasis on the phytopathogenic pseudomonads commonly found in California, and screened several cowpea varieties for their degree of resistance to *Pseudomonas syringae*.

MATERIALS AND METHODS.—Ten varieties of cowpea were inoculated with bacteria. The varieties included 'California Blackeye 3', 'California Blackeye 5', 'Queen Anne', 'Pinkeye Purple Hull', 'Bunch Purple Hull', 'Texas Cream 14', 'Mississippi Crowder' (P.I. 293535), 'Frisol Bayito' (P.I. 312208), P.I. 251222, and P.I. 339593. The origins of P.I. 251222 and P.I. 339593 were Afghanistan and South Africa, respectively. Only the variety California Blackeye 3 was used in all tests for all bacteria.

Cowpea seeds were germinated in a mixture of 500 g sand and 75 ml water. They were incubated in darkness and at alternating temperatures of 20 C for 16 hr and 30 C for 8 hr for 4 days, followed by 1 day in the light and at 23 C. The seedlings then were transplanted into a soil mixture of equal parts loam, sand, and peat to which was added 350 g of 16:16:16 fertilizer per $3.79 \times 10^{-2} m^3$ (10 gallons) soil. Four seedlings were planted in each 10.2-cm (4-inch) diam pot, and these were maintained on the greenhouse bench for 24 hr prior to inoculating them. The greenhouse was maintained at 32 C during the day and at 21 C at night.

Primary leaves were prepared for inoculation by first spraying them with distilled water and then dusting them with trace amounts of Carborundum. Two ml distilled water were added to 48-hr-old cultures grown on yeast-dextrose-calcium carbonate agar, and the resulting concentrated bacterial suspension (10^9-10^{10} cells/ml) was gently rubbed, with a cotton-tipped applicator, onto the leaves. Inoculated seedlings were covered with a plastic bag for 96 hr. Control plants (wound-inoculated with Carborundum and water) were included, and all plants were returned to the greenhouse bench. An alternate method of applying the bacterial suspension with an atomizer yielded the same results but cross-contamination of cultures was increased. Rubbing the leaves was selected because it enabled the rapid inoculation of large numbers of plants, the testing of numerous bacterial cultures at the same time, and it could be standardized. Preliminary tests revealed no difference in susceptibility between primary and trifoliate, and that is was necessary to inoculate young leaves.

Because numbers of bacteria applied to young leaves affected the length of time before symptoms appeared, readings were taken at 24 hr and 5 days after inoculation. Tests were not considered positive for infection unless all eight primary leaves showed brown necrotic or water-soaked lesions and/or chlorosis. Bruise injury was indicated as a negative result when only one or two leaves showed necrosis. Three trials were run for each bacterial culture.

Bacterial cultures.—Four-hundred, thirteen bacterial cultures were tested. These included 69 cultures of four *Agrobacterium* spp., 23 cultures of *Corynebacterium* spp., 55 cultures of 10 *Erwinia* spp., 214 cultures of 35 *Pseudomonas* spp., and 52 cultures of 12 *Xanthomonas* spp. The cultures were designated by an abbreviation of the name of the institution from which they were received, i.e., ICPB (International Collection of Phytopathogenic Bacteria), NCPPB (National Collection of Plant Pathogenic Bacteria), ATCC (American Type Culture Collection), UCBPP (University of California Berkeley Department of Plant Pathology), and UCDPP (University of California Davis Department of Plant Pathology). The cultures not designated as to source were from our laboratory. All the bacterial cultures were assigned State Laboratory (SL) accession numbers.

The *Agrobacterium* spp. tested were 51 isolates of *A. tumefaciens*, including ICPB-TT2, ICPB-TT3, ICPB-TT4, ICPB-TT5, ICPB-TT6, and 46 isolates from our laboratory; seven isolates of *A. radiobacter*, including ICPB-TR1, ICPB-TR4, ICPB-TR5, ICPB-TR6, ATCC-AR17, and two isolates from our laboratory; eight isolates of *A. rhizogenes*, including ICPB-TR7, ICPB-TR101, ICPB-TR102, ICPB-TR104, ICPB-TR105, and three isolates from our laboratory; three isolates of *A. rubi*, including ICPB-TR2, ICPB-TR3, and one isolate from our laboratory.

The *Corynebacterium* spp. tested were one isolate of *C. fascians*, one isolate of *C. flaccumfaciens*, one isolate of *C. insidiosum*, nine isolates of *C. michiganense*, two isolatses of *C. poinsettiae* from our laboratory; seven isolates of *C. sepedonicum*, including ICPB-CS117 and six isolates from our laboratory; and two of *C. rathayi*, ICPB-CR1 and ICPB-CR2.

The *Erwinia* spp. tested were 23 isolates of *E. amylovora* from our laboratory; two isolates of *E.*

aroideae, ICPB-EA3 and UCBPP-1; one isolate of *E. atroseptica* from our laboratory; eight isolates of *E. carotovora*, including UCDPP-3D31, UCBPP-1, UCBPP-2, and five isolates from our laboratory; four isolates of *E. chrysanthemi* including UCBPP-201, UCBPP-202, and two isolates from our laboratory; one isolate of *E. cypripedii* from our laboratory; three isolates of *E. quercina*, including UCBPP-2, UCBPP-3, and one isolate from our laboratory; six isolates of *E. nigrifluens* and six isolates of *E. rubrifaciens* from our laboratory; and one isolate of *E. carotovora* var. *parthenii*, ICPB-EC124. Only one isolate of *Escherichia coli*, UCDPP-9D3, was used.

The *Pseudomonas* spp. tested were one isolate of *P. aeruginosa*, UCDPP-2390; two isolates of *P. alliicola*, UCBPP-3 and UCBPP-5; one isolate of *P. andropogonis*, NCPPB-934; three isolates of *P. angulata*, including UCBPP-1, UCBPP-2, and UCBPP-3; two isolates of *P. cattleyae* from our laboratory; two isolates of *P. cepacia*, UCBPP-7 and UCBPP-10; one isolate of *P. chlororaphis*, UCBPP-31; four isolates of *P. cichorii*, including UCDPP-PC140, UCDPP-PC139, UCDPP-PC29, and UCDPP-26; one isolate of *P. coronafaciens* from our laboratory; 44 isolates of *P. eriobotryae* including Okabe's culture from Japan and 43 isolates from our laboratory; 14 isolates of *P. fluorescens*, including ATCC-I15, ATCC-I16, ATCC-I17, ATCC-I18, ATCC-I20, UCDPP-2632, UCBPP-2, UCBPP-33, UCBPP-108, UCBPP-143d, UCBPP-192, UCBPP-210, UCBPP-410, and UCBPP-411; four isolates of *P. glycinea* including UCDPP-PG102, UCDPP-PG108, UCDPP-PG2, and one isolate from our laboratory; five isolates of *P. lachrymans*, one isolate of *P. lapsa*, and two isolates of *P. maculicola* from our laboratory; three isolates of *P. marginalis* including UCBPP-400, UCBPP-401, and UCDPP-PM171; four isolates of *P. marginata* including ATCC-10247, UCBPP-11, UCBPP-188, and UCDPP-PM106; nine isolates of *P. mori* from our laboratory; seven isolates of *P. morsprunorum* including NCPPB-1095, UCDPP-PM1, UCDPP-PM2, UCDPP-PM3, UCDPP-PM4, UCDPP-PM6, and UCDPP-PM7; nine isolates of *P. phaseolicola*, including UCBPP-HB8, UCBPP-HB9, UCBPP-HB11, UCBPP-HB13, UCDPP-PP132, UCDPP-PP142, UCDPP-PP180, UCDPP-PP181, and UCDPP-PP185; two isolates of *P. pisi*, UCBPP-13 and UCBPP-17; two isolates of *P. polycolor*, UCBPP-2 and UCBPP-3; one isolate of *P. primulae* from our laboratory; seven isolates of *P. putida*, including ATCC-M6, ATCC-M7, ATCC-M8, ATCC-M9, ATCC-M10, UCBPP-A3-12, and UCDPP-2693; one isolate of *P. rubrilineans*, UCBPP-2; one isolate of *P. rubrisubalbicans*, UCBPP-1; one isolate of *P. setariae*, NCPPB-1392; 12 isolates of *P. savastanoi*, including ICPB-PS182, UCDPP-ID4, and 10 isolates from our laboratory; one isolate of *P. sesami*, NCPPB-1017; four isolates of *P. solanacearum*, including UCDPP-PS257, Buddenhagen B-139, Buddenhagen P-28-T, and Buddenhagen blue mutant; 45 isolates of *P. syringae*, including UCBPP-S71, UCDPP-5D425, UCDPP-5D443, UCDPP-5D446, UCDPP-5D463, UCDPP-5D457, UCDPP-5D480, UCDPP-5D498, UCDPP-5D4105, UCDPP-5D4205, and 35 isolates from our laboratory; one isolate of *P. tabaci*, UCBPP-2; three isolates of *P. tomato* from our laboratory; two isolates of saprophytic *Pseudomonas* spp.; and 12 isolates of phytopathogenic *Pseudomonas* spp. from different plant hosts from our laboratory.

The *Xanthomonas* spp. tested were three isolates of *X. campestris*, three isolates of *X. fragariae*, seven isolates of *X. hederae*, two isolates of *X. incanae*, 16 isolates of *X. juglandis*, 14 isolates of *X. malvacearum*, one isolate of *X. pelargonii*, one isolate of *X. phaseoli*, and one isolate of *X. vitians* from our laboratory; one isolate of *X. corylina* from L. W. Moore; two isolates of *X. vesicatoria*, including UCDPP-6D5 and one isolate from our laboratory; and one isolate of *Xanthomonas* sp. from tomato from our laboratory.

RESULTS.—Three basic types of responses of cowpea seedlings to the bacterial cultures were observed on the primary leaves of California Blackeye 3. These types were: chlorosis only, produced by one isolate of *P. syringae* (SL 4079); water-soaked appearance induced by two isolates of *P. alliicola*; and brown necrotic spots of various sizes on the inoculated leaves induced by the rest of the bacterial isolates which produced a positive response.

Only two of 51 isolates of *A. tumefaciens* (Table 1) caused a weak response on the cowpea leaves. All cultures of *A. radiobacter*, *A. rhizogenes*, and *A. rubi* gave no reaction. All 23 isolates of seven

TABLE 1. Responses of the 'California Blackeye 3' cowpea to selected species of *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*

Species	Ratio of isolates causing positive reaction[a] (No./Total tested)	Type of reaction[b]
Agrobacterium tumefaciens	2/51	N
Erwinia amylovora	8/23	N
Pseudomonas spp.		
Pathogenic		
P. alliicola	2/2	WS
P. cattleyae	1/2	N
P. cichorii	3/4	N
P. eriobotryae	2/44	N
P. pisi	2/2	N
P. syringae	45/45	N, C
Pseudomonas spp.[c]	4/12	N
Saprophytic[d]		
P. fluorescens	1/14	N
Xanthomonas vesicatoria	2/2	N

[a] Positive reaction indicated by development of brown necrotic spots on primary leaves at 24-48 hr after inoculation.

[b] N = necrosis; C = chlorosis; and WS = water-soaked spot.

[c] Greenish fluorescent pseudomonads that were negative for oxidase, arginine dihydrolase, and 2-ketogluconate tests, and positive for the hypersensitive reaction on tobacco.

[d] Greenish fluorescent pseudomonads that were positive for oxidase, arginine dihydrolase, and 2-ketogluconate tests, and did not cause hypersensitive reaction on tobacco.

TABLE 2. Types of reaction observed on 10 cowpea varieties 5 days after they were inoculated with 27 isolates of *Pseudomonas syringae* obtained from various hosts

Host (isolate No.)	Cowpea reaction[a]		
	SR	WR	NR
Apple			
4152	0	2	8
Apricot			
4074,4130,4133,4135	10	0	0
Cherry			
4081, 4136	10	0	0
Chrysanthemum			
4049	8	0	2
Citrus			
4014	9	0	1
4019	10	0	0
4075	7	1	2
Lilac			
4016, 4079	10	0	0
Liquidambar			
4150	10	0	0
Nectarine			
4080	5	0	5
Oleander			
4134, 4141, 4149	10	0	0
4137	3	0	7
4142	4	2	4
Peach			
4073	5	3	2
4078	10	0	0
Pear			
4146	10	0	0
Pepper			
4082	8	0	2
Plum			
4076	9	0	1
Pyracantha			
4159	4	0	6
Walnut			
4077	9	0	1

[a] SR = strong necrotic reaction; WR = weak necrotic reaction; and NR = no reaction.

Corynebacterium spp. exhibited a negative reaction on cowpea leaves within 5 days of inoculation, and observation until the 10th day still revealed no reaction. Therefore, the reaction of cowpea to all these cultures was designated negative.

Within the *Erwinia* group, only eight of 23 isolates of *Erwinia amylovora* caused brown necrotic spots on cowpea leaves at 48 hr after inoculation. These eight isolates, designated SL 3037, 3039, 3052, 3053, 3054, 3055, 3060, and 3061, were recovered from cherry (*Prunus avium*), pear (*Pyrus communis*), apple (*Malus sylvestris*), *Cotoneaster* sp., *Pyracantha* sp., hawthorn (*Crataegus oxyacantha*), and Indian hawthorn (*Raphiolepis indica*), respectively.

Pseudomonas spp. behaved differently from those of other genera. The seedlings inoculated with certain species of *Pseudomonas* generally reacted rapidly and had developed brown necrotic spots on the primary leaves 24 hr after inoculation. The pseudomonads that induced positive responses on cowpea were: two isolates of *P. alliicola*; one of two isolates of *P. cattleyae*; two of 44 isolates of *P. eriobotryae*, including the Japanese culture and strain B (9) of California; two isolates of *P. pisi*; all 45 isolates of *P. syringae*; three of four isolates of *P. cichorii*; four of 12 isolates of pathogenic *Pseudomonas* spp. recovered from Boysenberry (*Rubus* sp.) SL 4171, English ivy (*Hedera helix*) SL 4170, persimmon (*Diospyros virginiana*) SL 4144, and strawberry (*Fragaria chiloensis*) SL 4172, respectively; and one of 14 isolates of *P. fluorescens*.

Only two isolates of *Xanthomonas vesicatoria* produced tiny discrete necrotic spots on the primary leaves 48 hr after inoculation. No other isolates of *Xanthomonas* spp. caused any response.

Response of cowpea varieties to P. syringae.—The cowpea variety, California Blackeye 3, responded to all 45 cultures of *P. syringae*, but not to *P. morsprunorum*, regardless of the host and locations from which they were obtained (Table 1). Therefore, 10 different cowpea varieties were selected to test their responses to the 27 individual cultures of this bacterium. Thirteen (SL 4016, 4019, 4074, 4078, 4130, 4133, 4134, 4135, 4136, 4141, 4146, 4149, and 4150) of 45 isolates caused brown necrotic spots on all tested varieties, and three (SL 4014, 4076, and 4077) produced necrotic brown lesions on nine varieties (Table 2). Isolate SL 4079 produced chlorotic spots on varieties California Blackeye 3 and California Blackeye 5, in addition to brown necrotic spots on the other eight varieties. The reaction with isolate SL 4152 from apple was weakly positive on California Blackeye 3 and California Blackeye 5, but negative on each of the other eight varieties. The degrees of varietal susceptibility to all 27 isolates indicated that California Blackeye 3 is most susceptible (27/27); Texas Cream 14 (24/27), Frisol Bayito (25/27), and P.I. 251222 (25/27) are susceptible; and Pinkeye Purple Hull (18/27) and P.I. 339593 (20/27) are less suceptible. No variety except California Blackeye 3 exhibited a positive reaction to all *P. syringae* cultures.

DISCUSSION.—Our results (Table 1) indicate that the cowpea variety, California Blackeye 3, can be used as an aid in testing isolates of *P. syringae* because 45 isolates were used and they all gave positive reactions. However, the number of isolates of *P. alliicola, P. cattleyae, P. cichorii, P. pisi*, and *X. vesicatoria* are so small that definite conclusions cannot be made. For example, the two isolates of *P. alliicola, P. pisi*, and *X. vesicatoria* may represent in their respective groups, the two of 44 isolates of *P. eriobotryae* which gave positive reactions. Layne (10) indicated that cowpea can be used as a laboratory test plant for *E. amylovora*. However, certain necrotic reactions on cowpea were obtained with eight of 23 isolates of *E. amylovora* used in this study. The reason for positive reactions for two of 51 isolates of *A. tumefaciens* is not known. Cowpea is not useful for testing any *Corynebacterium* sp.

The hypersensitive test (8) on tobacco leaves generally has been utilized for identifying phytopathogenic pseudomonads, but it has not been

adapted to detect interspecific differences. We found that the cowpea inoculation test was a useful supplementary method for identifying 45 isolates of *P. syringae*. Stapp (20) and Fuchs (3) indicated that *P. morsprunorum* resembles *P. syringae* so closely that the former cannot be considered separate species. The data in Table 1 indicate that by using cowpea variety California Blackeye 3, we can differentiate *P. syringae* from other greenish fluorescent phytopathogenic pseudomonads. The data reported herein indicate it is impossible to identify fluorescent phytopathogenic pseudomonads solely on the results of inoculation of cowpea. Additional tests such as the oxidase (13, 17), arginine dihydrolase (17), and host pathogenicity tests are necessary to differentiate the pathogens.

The following routine tests which would be used to identify *P. syringae* in our laboratory are (i) negative reactions for arginine dihydrolase (17) and oxidase (13, 17); (ii) positive hypersensitive reaction on tobacco (8); (iii) liquifaction gelatin (1); (iv) negative reaction for pectolytic enzyme production on pectin and pectate gels (5); and (v) production of necrotic spots on the primary leaves of California Blackeye 3 cowpea.

The selection of indicator plants for bioassay of *P. syringae* is an important factor, because cowpea varieties differ in their response to different isolates of *P. syringae* (Table 2). Therefore, if the proper variety of cowpea is not used, a misleading response might occur.

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