Detection of Pathogenicity, Measurement of Virulence, and Determination of Strain Variation in *Pseudomonas syringae* pv. *syringae*

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ABSTRACT

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Virulence among strains of *Pseudomonas syringae* pv. *syringae* was compared by leaf and twig inoculation of peach trees (*Prunus persica*) in the greenhouse. Immature cherry fruit and pear, apple, and peach seedlings were also tested as bioassays for pathogenicity and virulence. Lesions induced on cherry fruit correlated poorly with lesions on potted peach trees in the greenhouse, but cherry fruit were capable of detecting pathogenicity in moderately to highly virulent strains. Etiolated pear and apple hypocotyls responded to differences in virulence among strains and showed high correlations with peach tree inoculations in the greenhouse. A bioassay using peach seedling cotyledons was developed for detecting pathogenicity among strains from several host sources and pathovars. Foliar inoculation of peach, apricot, nectarine, and plum cultivars with three *P. syringae* pv. *syringae* strains from stone fruit hosts indicated that these strains varied more in levels of virulence than in ability to infect specific hosts.

Additional key words: bacterial canker, *Prunus* spp.

Among the pathovars of *Pseudomonas syringae* known to infect stone fruits, *P. syringae* pv. *syringae* has shown less specificity for the original host than has *P. syringae* pv. *morsprunorum* (5). Many woody and herbaceous hosts are susceptible to at least one strain of these two pathovars (2). Within peach orchards, heterogeneous populations of *P. syringae* pv. *syringae* exist whose individual members vary in biochemical characteristics (7). Individual cells within one strain may also vary in virulence (14) and a single strain may induce various symptoms on one host or cause more than one infection phase per disease cycle (17). Such heterogeneity, combined with wide host ranges (1), may be important for cross-inoculation within orchards (16).

The necessity for screening large numbers of bacterial isolates has resulted in numerous studies to identify easily measurable traits that correlate with levels of virulence. Such traits as biochemical properties (2,8,11,12,16), phage sensitivity (4,8), and serological relationships (1,8,13,14) were shown to be more closely associated with geographic or host origins than with virulence. The ability to produce toxin has been correlated with virulence (6,9), but its detection in vitro has met with varying degrees of success (8). An alternative approach is to inoculate detached plants or plant parts as bioassay indicators. Puncture inoculations of immature pear, cherry, and lemon fruit have been used to separate stone fruit strains from pear, citrus, and lilac strains (8). Distal internodes of peach seedlings were useful in pathogenicity tests because of their susceptibility to pathogenic strains regardless of host origin (13). Strains have been ranked, to a limited extent, by differences in virulence using necrotic reactions on Red Kidney bean pods (14). A hypocotyl inoculation technique on apple seedlings was used to detect pathogenicity in *Erwinia amylovora* strains and showed promise for use with *P. syringae* (15).

The objectives of this study were 1) to develop and compare bioassays for detection of pathogenicity and measurement of virulence in *P. syringae* pv. *syringae*, 2) to evaluate the ability of these bioassays to predict virulence in peach trees by correlating the test results with greenhouse data, and 3) to determine the variability in expression of virulence by selected strains when inoculated onto various *Prunus* spp. and cultivars.

MATERIALS AND METHODS

Strains. Bacterial isolates were initially classified as *P. syringae* pv. *syringae* on the basis of their oxidase reaction, production of fluorescent pigment, arginine metabolism, gelatin liquefaction, aesculin hydrolysis, and hypersensitive reaction in tobacco (10,11). Inoculation

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Table 1. Strains of *Pseudomonas syringae* used for inoculation and their corresponding lesions induced on bioassay indicators and peach trees

P. syringae pathovar Strain	Original host	Bioassay results (± SD)[a] Mean lesion diam., cherry fruit (mm)	Mean lesion length, pear hypocotyl (mm)	Mean lesion length, apple hypocotyl (mm)	Mean lesion diam., peach cotyledon (mm)	Greehouse peach inoculations (± SD) Mean no. lesions/leaf	Mean canker length (mm)
pv. *syringae*							
B-3A	Peach	···	···	···	3.4 ± 0.8	7.1 ± 1.3	5.7 ± 2.0
B-15+	Almond	5.2 ± 2.2	19.0 ± 3.6	17.8 ± 2.5	2.4 ± 0.9	5.4 ± 0.6	7.2 ± 1.9
11-A	Apricot	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
AP-1T	Apricot	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PS-1	Peach	4.2 ± 3.0	6.2 ± 1.3	5.9 ± 1.6	2.3 ± 0.9	3.9 ± 2.3	1.2 ± 0.7
PS-3	Peach	3.2 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	0.6 ± 0.7	0.0 ± 0.0
PS-8	Peach	3.2 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	0.6 ± 0.5	0.0 ± 0.0
PS-10	Peach	6.0 ± 0.8	6.5 ± 1.7	5.9 ± 1.8	2.3 ± 0.8	3.9 ± 2.3	1.3 ± 0.6
PS-14	Apricot	2.5 ± 0.6	8.2 ± 11.2	10.4 ± 11.2	2.2 ± 0.8	1.8 ± 1.2	3.5 ± 1.3
PS-23	Cherry	···	0.0 ± 0.0	3.9 ± 2.0	2.9 ± 0.6	1.0 ± 0.8	0.8 ± 0.9
pv. unknown							
PS-18	Walnut	2.9 ± 1.4	0.8 ± 1.0	3.0 ± 1.4	3.0 ± 1.0	1.9 ± 0.2	1.3 ± 0.6
PS-19	Walnut	2.8 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	0.3 ± 0.4	0.0 ± 0.0
Ma.2	Maple	···	1.0 ± 1.1	3.9 ± 0.8	1.4 ± 0.6	0.5 ± 0.4	0.2 ± 0.3
Ma.11	Maple	···	1.5 ± 0.9	6.2 ± 3.7	2.7 ± 1.0	1.1 ± 0.2	1.2 ± 0.8
pv. *papulans*	Apple	0.0 ± 0.0	0.8 ± 1.1	2.5 ± 3.8	0.8 ± 0.2	0.1 ± 0.1	0.2 ± 0.2
pv. *tabaci*	Tobacco	0.0 ± 0.0	0.6 ± 0.9	2.4 ± 2.0	0.7 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
pv. *tomato*	Tomato	0.0 ± 0.0	0.8 ± 0.8	3.1 ± 2.5	1.2 ± 0.6	0.3 ± 0.2	0.3 ± 0.5

[a]Standard deviation.

Table 2. Mean number of lesions per leaf resulting from inoculation of three strains of *Pseudomonas syringae* pv. *syringae* onto various *Prunus* cultivars

Host species Cultivar	Inoculum strain[x] B-15+	PS-10	PS-14
Peach (*P. persica*)			
Fairtime	15.7[y]a[z]	7.0 a	4.3 a
Loring	11.8 b	6.9 a	4.5 a
Elberta	10.3 bc	5.2 a	2.5 b
Redhaven	8.3 c	6.6 a	4.3 a
Nectarine (*P. persica*)			
Fantasia	10.0 bc	5.8 a	4.9 a
Apricot (*P. armeniaca*)			
Blenvil	3.1 de	0.4 b	0.1 c
Sundrop	3.3 de	0.1 b	0.1 c
Plum (*P. domestica*)			
Morris	2.2 de	0.1 b	0.1 c
Stella	1.9 de	0.0 b	0.0 c
Frontier	1.3 de	0.3 b	0.4 c
Wade	0.8 e	0.0 b	0.0 c
Santa Rosa	0.7 e	0.0 b	0.0 c
LSD	3.5	1.8	0.9

[x]B-15+ isolated from almond, PS-10 isolated from peach, and PS-14 isolated from apricot.
[y]Means are based on three to six replicates and four to 10 subsamples per replicate.
[z]Means within the same column followed by the same letter were not significantly different at $\alpha = 0.05$ according to Fisher's least significant difference test.

tests used two groups of strains: The first group consisted of 10 wild-type strains of *P. syringae* pv. *syringae* varying in host origin (Table 1) and included strains B-3A and B-15+ (6). The second group included seven strains from other pathovars of *P. syringae* (pv. *papulans, pisi, tabaci,* and *tomato*), and strains of *P. syringae* isolated from non-stone fruit hosts (Table 1). All strains within the second group except PS-19 were shown to be pathogenic on their host of origin.

Fruit bioassays. Immature cherry fruit (cultivars Schmidt and Windsor) were surface-disinfested in 0.52% sodium hypochlorite and arranged in moist chambers. Inoculation techniques were modified from those described earlier (8,10). Using a sterile transfer needle, fruit surfaces were punctured about 2 mm deep. Thirty-six-hour cultures of each test inoculum (grown on nutrient agar at 24 C) were suspended to 10^7 cfu/ml in sterile phosphate buffer (pH 7.0, 0.02 M), then introduced to the punctured surface as individual droplets using a sterile transfer loop. Lesion diameters were measured after 1 wk of incubation at room temperature (20–22 C). Inoculations of two fruits per strain were repeated over time to total four to six replicates.

Seedling bioassays. Apple and pear seeds (cultivars Jonathan and Bradford, respectively) were prepared by a previously described method (15). Seeds were stratified at 3 C, disinfested in 0.52% sodium hypochlorite, and refrigerated in moistened, sterile vermiculite. Germinated seedlings were arranged in dissecting trays lined with moistened paper towels, covered with aluminum foil, and allowed to grow at room temperature (20–22 C) for 1–3 days. The etiolated hypocotyls were puncture-inoculated 1 cm below the cotyledons with a 23-gauge needle that had been touched to several colonies of a 36-hr nutrient agar culture. The trays were then enclosed in plastic bags. Lesion lengths were measured after 4–5 days of incubation. Inoculations of two seedlings per strain were repeated over time to total four to six replicates.

Peach seeds (cultivar Lovell) were removed from their endocarps and stratified in moist perlite at 3 C for 12 wk. Seedlings were then germinated in moistened vermiculite at room temperature. Puncture inoculations followed the same procedure as for apple and pear seedlings, except the needle was inserted in the center of each cotyledon and extended through to the opposite surface. Lesion diameters were measured on both the adaxial and abaxial cotyledon surfaces after 4 or 5 days of incubation at room temperature and were averaged for both cotyledons. Inoculations were repeated over time to total four to six replicates.

Greenhouse inoculations. One-year-old peach, apricot, and plum trees of several varieties (Table 2) were brought out of dormancy in the greenhouse. New shoots about 20 cm long were inoculated on both leaf surfaces by applying a 10^7-cfu/ml bacterial suspension with a sterile cotton-tip applicator. Inoculations were replicated on three to six trees per variety. Twigs of inoculated leaves were individu-

ally enclosed in plastic bags for 48 hr, then exposed to normal greenhouse conditions (range 20–36 C) for another 5–7 days. Results for each twig were recorded as the mean number of lesions per leaf for each of four to 10 inoculated leaves.

Twigs were inoculated by excising the petiole at the point of attachment to the twig with a sterile scapel and applying a drop of 10^7-cfu/ml bacterial suspension to the wound surface. Canker lengths were measured longitudinally along the twig after 10–14 days of incubation. The first, third, and fifth fully expanded leaves were selected as inoculation sites and averaged to account for variations in tissue age and response. Inoculations were replicated on three to six trees per variety.

RESULTS

Detection of pathogenicity and measurement of virulence. All fruit and seedling bioassays were capable of identifying pathogenic strains of *P. syringae* pv. *syringae*. Cherry fruit inoculated with pathogenic strains of *P. syringae* pv. *syringae* developed black, sunken lesions 2–8 mm in diameter that were easily distinguished from the dry punctures or slightly reddish halos formed in response to nonpathogenic strains and to strains from other pathovars. Symptoms on apple and pear seedlings inoculated with *P. syringae* pv. *syringae* strains ranged from short, scabby lesions to extensive rots of the entire hypocotyl and epicotyl (Fig. 1, Table 1). The extent of lesions on these seedlings was related to the relative level of virulence of the bacterial strain. Weakly virulent strains and strains originating from hosts other than stone fruits induced small, well-delineated lesions (Fig. 1). Control inoculations using a sterile needle induced a slight discoloration around the puncture site (Fig. 1).

Inoculated peach seedlings developed circular, dark brown, sunken lesions on both cotyledon surfaces (Fig. 2). Their response differed from that in pear and apple hypocotyls in that the peach seedlings were less selective in relation to pathovar or host origin (Fig. 2). Highly virulent strains produced larger lesions, regardless of host origin. Weakly virulent and nonpathogenic strains induced only a slight darkening at the puncture site, sometimes accompanied by faint chlorotic halos, which could not be distinguished from control punctures (Fig. 2).

A pathogenic strain of *E. amylovora* was included in all bioassays. Reactions to this pathogen were easily distinguished from those to *P. syringae* and consisted of water-soaked rots and formation of bacterial ooze droplets within 24 hr; these did not occur with *P. syringae*.

Variation in virulence among strains. Mean lesion sizes induced by each strain on each of the bioassay indicators were subjected to correlation analysis. The analysis indicated that the results of one bioassay generally did not compare closely with the results of another (Table 3). Pear and apple hypocotyl inoculations produced the most similar results (r = 0.94, $P<0.01$). Data from these bioassays also correlated strongly with leaf and stem lesions on potted peach trees (Table 4), whereas cherry fruit bioassays correlated weakly with leaf infections but not with canker length. The cotyledon bioassay using peach seedlings correlated only weakly with potted tree data.

Strain × host interactions. Three strains of *P. syringae* pv. *syringae*, isolated from almond, peach, and apricot, were inoculated onto shoots of various *Prunus* cultivars in the greenhouse (Table 2). Because all *Prunus* spp. responded to foliar inoculation but cankers were induced only in *P. persica*, the mean number of lesions per leaf was chosen as the test criterion.

Morphology of foliar lesions varied with *Prunus* spp. and cultivars. Lesions were beige to tan, with reddish to brown margins, and sometimes bounded by chlorotic halos. On more susceptible hosts, such as *P. persica* cultivars, lesions were irregular in shape and often coalesced. Necrotic areas eventually dropped from the lamina, leaving shot-hole symptoms.

Peach and nectarine cultivars were more susceptible to foliar infection than were apricots or plums (Table 2). Plum

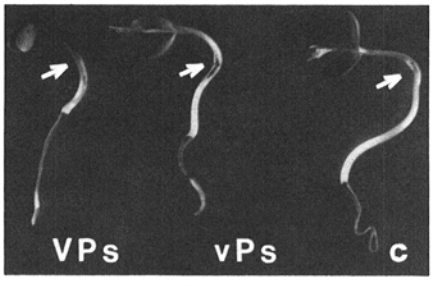

Fig. 1. Response of apple seedlings (cultivar Jonathan) to hypocotyl inoculation with a highly virulent strain of *Pseudomonas syringae* pv. *syringae* (VPs) and a weakly virulent strain (vPs); c = uninoculated control. Arrows indicate inoculation sites.

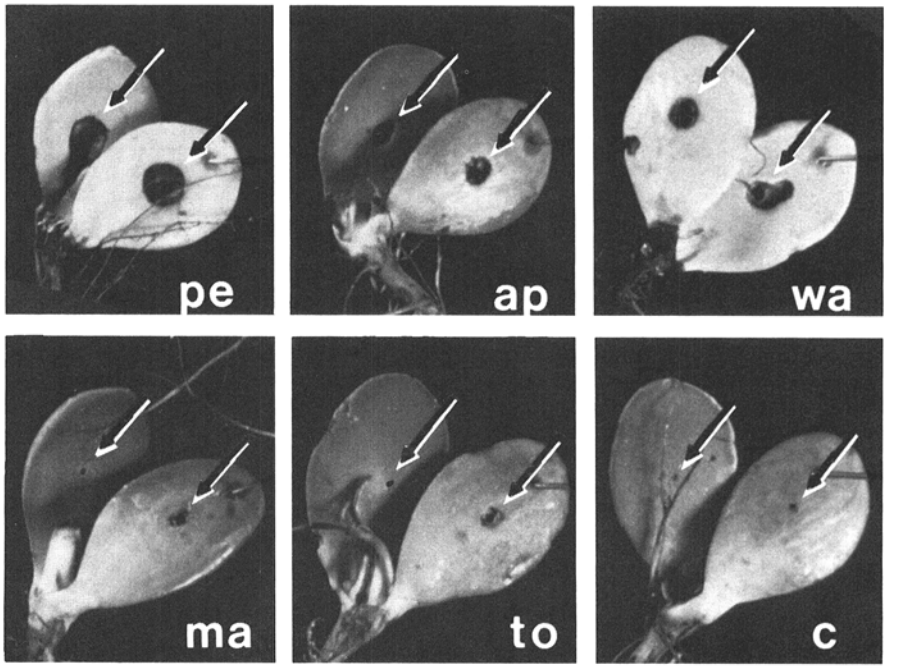

Fig. 2. Response of peach cotyledons to puncture inoculation with *Pseudomonas syringae* strains from peach (pe), apricot (ap), walnut (wa), maple (ma), and tomato (to); c = uninoculated control. Arrows indicate inoculation sites.

Table 3. Pearson's correlation coefficients for lesion sizes resulting from inoculation of *Pseudomonas syringae* pv. *syringae* strains onto various bioassay indicators

	Jonathan apple hypocotyl	Bradford pear hypocotyl	Cherry fruitlet	Lovell peach cotyledon
Jonathan apple hypocotyl	...	0.94 ($P<0.01$)	0.45 ($P=0.36$)	0.54 ($P=0.09$)
Bradford pear hypocotyl		...	0.56 ($P=0.24$)	0.54 ($P=0.09$)
Cherry fruitlet			...	0.35 ($P=0.49$)
Lovell peach cotyledon				...

Table 4. Correlation of virulence in peach trees to performance on bioassay hosts (lesion length [mm]) inoculated with strains of *Pseudomonas syringae*

Bioassy indicator	Pearson's correlation coefficients	
	Peach leaf necrosis (no. lesions/ leaf)	Peach stem cankers (length, mm)
Jonathan apple hypocotyls	0.84 ($P < 0.01$)	0.69 ($P = 0.03$)
Bradford pear hypocotyls	0.92 ($P < 0.01$)	0.62 ($P = 0.06$)
Lovell peach cotyledons	0.51 ($P = 0.11$)	0.53 ($P = 0.07$)
Cherry fruitlets	0.77 ($P = 0.07$)	−0.03 ($P = 0.95$)

leaves were generally poor hosts for *P. syringae* pv. *syringae*. Bacterial strains also varied in levels of virulence, B-15+ being most virulent on all cultivars. Analylsis of variance indicated that the variation in the number of lesions was mainly determined by the bacterial strain, such that each strain showed the same relative level of virulence from host to host (Table 2). No species or variety specificities were detected.

DISCUSSION

When selecting a bioassay for *P. syringae* pv. *syringae*, the following objectives should be considered: 1) detection of pathogenicity, 2) measurement of levels of virulence, and 3) prediction of virulence under greenhouse or field conditions. Peach cotyledons, for example, were capable of distinguishing pathogenic strains from a wide range of hosts and are thus well suited as a bioassay for detecting pathogenicity. Cherry fruit were also capable of detecting pathogenicity but only in *P. syringae* pv. *syringae* and walnut strains PS-18 and PS-19, and with less sensitivity. These walnut strains were biochemically and pathogenically similar to *P. syringae*. In contrast, apple and pear hypocotyls responded to varying degrees of virulence in *P. syringae* pv. *syringae* and were also of predictive value for peach stem and leaf infections on potted trees (Table 4).

Several problems can be associated with bioassays for determining pathogenicity. First, use of large inoculum concentrations may produce diseaselike symptoms that may not represent natural pathogenic ability (12). Such artifacts were avoided in our study by interpreting a bioassay lesion as an indication of inherent pathogenicity rather than a demonstration of pathogenic capability on the plant species used as the bioassy indicator. At the other extreme, the bioassay may not be sufficiently sensitive to distinguish between saprophytes and weak pathogens (12). This insensitivity was encountered in the cherry fruit bioassay when used to compare *P. syringae* strains of other pathovars. These bioassays, with the exception of the peach cotyledons, should thus be restricted to stone fruit strains of *P. syringae* pv. *syringae*. The peach cotyledon bioassay was more sensitive in distinguishing pathogenic *P. syringae* strains from non-stone fruit hosts and is applicable to a wider range of isolates without the risk of creating artifacts.

We report for the first time the inoculation of peach seedling cotyledons as a sensitive bioassay for detecting pathogenicity in strains of *P. syringae* pv. *syringae*. This bioassay was capable of detecting pathogenicity in strains from other hosts and in strains of other *P. syringae* pathovars as well.

Some authors advocate a lack of specificity in *P. syringae* pv. *syringae* (1,16), whereas others disagree (3,17,18). Potted tree inoculations in our study (Table 2) did not detect host-specific interactions. Our data support the hypothesis that most variability among stone fruit-infecting strains of *P. syringae* pv. *syringae* is due to differences in levels of virulence rather than in ability to infect specific hosts.

None of the *P. syringae* pv. *syringae* strains isolated from stone fruit trees in North Carolina during 1981–1983 approached the high levels of virulence shown by the California strains B-3A and B-15+. These latter two strains are considered highly and moderately virulent, respectively (6). Although highly virulent strains may exist in North Carolina orchards, the predominance of weakly virulent strains may help explain the low incidence of bacterial canker of peach trees in this area.

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