A Detached-Leaf Bioassay for *Xanthomonas campestris* pv. *pruni*

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Accepted for publication 18 April 1985.

ABSTRACT

Randhawa, P. S., and Civerolo, E. L. 1985. A detached-leaf bioassay for *Xanthomonas campestris* pv. *pruni*. Phytopathology 75:1060-1063.

Young leaves of peach seedlings were detached and surface sterilized with 70% ethanol. Inoculum (10^6 colony-forming units [cfu] per milliliter) of *Xanthomonas campestris* pv. *pruni* was infiltrated with a needle-less syringe at several sites (approximately 70 cfu per site) on the abaxial side. The leaves were incubated on 0.5% water agar for 2 wk at 25 C under a 16-hr photoperiod. Leaves remained healthy and disease symptoms developed at the inoculation sites. The number of lesions produced at each site was directly proportional to the amount of inoculum infiltrated. Symptom expression and incubation time on detached leaves were similar to those on attached leaves. Symptoms specific for *X.c.* pv. *pruni* were not observed with 51 saprophytic bacterial strains tested at inoculum concentrations as high as 1×10^8 cfu/ml. At inoculum concentration of 1×10^6 cfu/ml, the detached leaf response distinguished the previously reported differential field resistance reactions of 21 of 22 peach cultivars.

Additional key words: bacteria, disease resistance, pathogenicity, techniques.

Xanthomonas campestris pv. *pruni* is the cause of bacterial spot disease of peach, apricot, plum, and cherry. The bacterium naturally enters leaves through stomata (10,16). Bacterial suspensions are normally atomized onto leaves and high humidity is maintained for 24–48 hr to artificially induce leaf infection within 10–14 days at 27 C (14,16). Our attempts (*unpublished*) to reproduce the disease on greenhouse-grown seedlings were frequently unsuccessful, as reported earlier (6). This inconsistency may depend upon oxygen availability in the root zone, plant nutrition, and the time of day when inoculations are made (11,12). These conditions directly influence related factors such as size of stomatal openings, water congestion, and infection on peach leaves (11). However, inoculations of leaves with *X.c.* pv. *pruni* by spraying inoculum at 1.25×10^{-1} kg/cm^2 pressure onto defined circular areas on leaves (3), wind driven rains (5), and by injection into plant tissue with a hypodermic syringe (14) consistently produced infections. The first method (3) delivers a defined amount of inoculum into leaf tissue. However, it is laborious and unsuitable when a large number of pathogen strains or host cultivars are to be screened. Alternatively, detached plant parts have been used to detect pathogenicity. These include stone fruits for *X.c.* pv. *pruni* (7) as well as lemon fruits, bean pods, potato slices, carrot disks, and tomato and pepper leaves for other bacterial plant pathogens (1,2,7,9,15). Inoculation of detached pepper and tomato leaves inoculated with suspensions of *X. campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* and incubating them on water agar resulted in multiplication of these bacteria within leaves from undetectable levels to 10^6–10^7 colony-forming units (cfu) per gram in 96 hr (15). In addition, typical disease symptoms were produced. Use of detached leaves has the added advantage that they can easily be handled and manipulated easily under laboratory conditions.

The purpose, therefore, of this study was to develop a detached-leaf bioassay for pathogenicity of *X.c.* pv. *pruni* on peach. A preliminary report has been published (13).

MATERIALS AND METHODS

Leaves. Greenhouse-grown peach (cultivar Sunhigh) seedlings were routinely used. One-year-old seedlings growing in 30.5-cm (12-inch)-diameter pots were pruned to allow new shoots to develop. The leaves that developed were more tender than the leaves on field-grown Sunhigh peaches. Young, fully expanded leaves (e.g., third to sixth leaf from shoot tip) were collected in a plastic bag and brought to the laboratory. Larger leaves were cut into proximal and distal halves. The leaves or leaf portions were briefly washed under running tap water to remove dirt. After surface sterilization with 70% ethanol for 40–60 sec at room temperature (17), the leaves were rinsed with sterile distilled water. These leaves were immediately used for inoculations.

To evaluate differential resistance of peach cultivars to *X.c.* pv. *pruni*, 3- to 4-yr-old trees growing at the USDA Appalachian Fruit Research Station at Kearneysville, WV, were selected. Several young leaves from four trees of each of 22 cultivars were detached and pooled. These leaves were washed for at least 5 min under tap water and sterilized with 70% ethanol for 90 sec. Nine randomly selected leaves replicated three times were used for inoculation.

To compare attached and detached leaves, two young shoots on each of five Sunhigh seedlings growing in the greenhouse were selected. All leaves starting from the third leaf from the terminal to the 14th leaf on a shoot were tested. This also allowed comparison between younger and older leaves. Each leaf was cut in the middle with scissors to detach the distal half. The proximal half remained attached on the plant. Cutting leaves in half did no other apparent harm; the young attached half leaves continued to grow and expand normally. These attached leaf halves were inoculated and incubated in the greenhouse at 27 C, whereas the detached distal halves were individually washed, surface sterilized, and prepared for inoculation as described above.

Inoculation. *X.c.* pv. *pruni* strains XP-1, 84-3-2, and 84-6-2 were used. These strains were different in their plasmid profiles (*unpublished*). The bacteria were grown on nutrient agar containing 0.25% glucose (NGA) for 24–48 hr and a loopful of growth suspended in sterile water and adjusted to $A_{535\ nm} = 0.1$. The suspension contained 10^8 cfu/ml as determined by dilution plating on NGA. This inoculum, serially diluted 100-fold in sterile distilled water, was used to inoculate detached and attached leaves. Two methods of inoculation were compared. In the first method of inoculum infiltration, the detached leaves or leaf portions were placed abaxial side upward on three or four layers of sterile paper towel. Inoculum, held in a plastic 3-ml syringe without a needle, was infiltrated by applying gentle and steady pressure while holding the open end of the syringe against the leaf until a 2- to 4-mm-diameter area of mesophyll tissue was water-soaked. The attached leaves were supported by hand to facilitate infiltration. On one leaf or leaf portion, 8–10 sites approximately 1 cm apart were

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inoculated. During infiltration, the inoculum was unavoidably splashed onto other parts of the leaf. The leaves, therefore, were lightly blotted to remove excess inoculum. To determine the number of cfu infiltrated at each site, 100 sites on five leaves were inoculated as described above. The inoculated leaves were dried in the laminar-flow hood till water-soaking disappeared. The leaves were then surface sterilized with 1% sodium hypochlorite containing 0.2% sodium dodecyl sulfate for 3 min. This treatment removes bacteria from leaf surfaces without toxicity to bacteria present within leaf tissue (*unpublished*). After five rinses with sterile water, the leaves were ground in 10 ml of water in a mortar with a pestle. The number of cfu in the homogenate was determined on NGA (20). Approximately 70 cfu were infiltrated at each site.

In the second method of surface inoculation, 10-μl droplets of the bacterial suspension were placed at each site on the abaxial surface of detached leaves. To inoculate attached leaves by surface inoculation method, a young shoot was bent horizontally along a flat tray and held in place by several pieces of paper tape. Selected leaves were held abaxial side upward with the help of tape. The plant was positioned to minimize constraint on the shoot. The ends of the tray were cotton lined and a plastic cover placed to make a humidity chamber. Several layers of wet paper towel were placed in the chamber to maintain high humidity. The plastic cover could be placed or removed conveniently without disturbing the shoot and the leaves. Therefore, inoculations could be made precisely at selected sites. The experiment was conducted in the growth room at 25 C. Five leaves, replicated three times, were inoculated.

The inoculated detached leaves (inoculated sides up) were placed on 0.5% water agar (15–20 ml per 100 × 15-mm plastic petri dish) and incubated for up to 2 wk at 25 C under fluorescent lights (60–75 $\mu E \cdot s^{-1} \cdot m^{-2}$) timed to a 16-hr photoperiod. Incorporation of cycloheximide (200 μg/ml), kasugamycin (16 and 32 μg/ml) into the water agar, and incubation of the leaf segments at 22 or 28 C under light or dark conditions were also evaluated for effects on leaf health, symptom development, and suppression of contaminants.

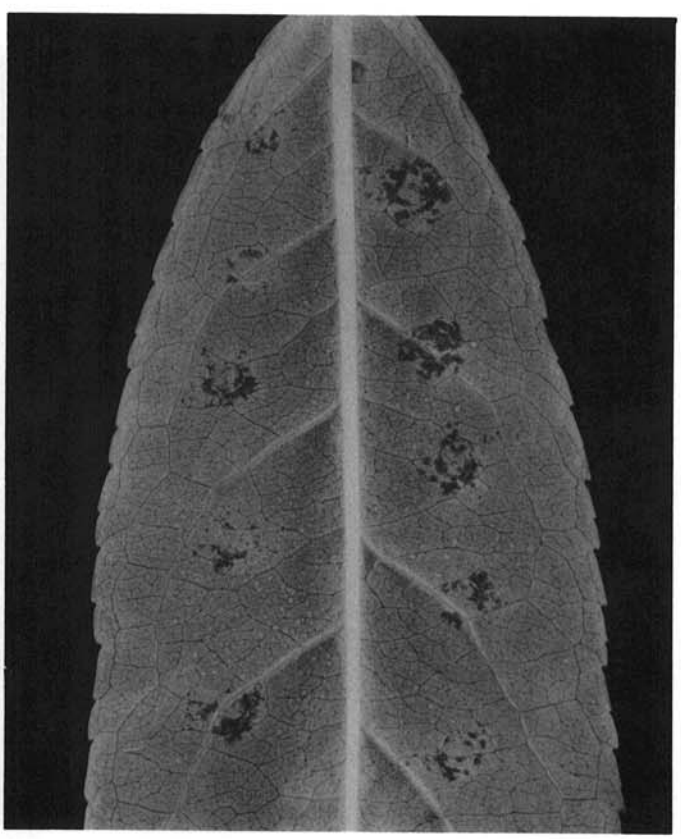

Fig. 1. Symptoms of bacterial spot disease on a detached peach leaf. Inoculum (1×10^6 cfu/ml) of *Xanthomonas campestris* pv. *pruni* infiltrated with a needle-less syringe into the leaf produced a 2- to 4-mm-diameter water-soaked area at each of 10 sites on the abaxial leaf surface. Following infiltration with inoculum, the leaf was incubated on 0.5% water agar for 2 wk.

Leaf response specificity. The reactions of detached leaves following infiltration with mixtures or single strains of saprophytic bacteria, epiphytically associated with peach and apricot leaves, were studied. Bacterial mixtures were obtained by washing peach leaves collected from four commercial orchards in New Jersey. The bacterial population in the washings varied from 8×10^3 to 8×10^4 cfu/ml. The absence of *X.c.* pv. *pruni* in the washings was verified by plating on selective medium (4). Fifty-one bacterial strains were isolated on NGA. Inoculum from 24- to 28-hr-old cultures on NGA was suspended in water, adjusted to $A_{535\ nm} = 0.1$ and presumed to contain approximately 1×10^8 cfu/ml. The inoculum was serially diluted 100-fold and infiltrated into detached leaves as described above.

RESULTS

Leaf health and disinfestation. Leaves and leaf portions placed on water agar remained healthy for at least 2 wk. On the other

TABLE 1. Detached peach cultivar Sunhigh leaf response to *Xanthomonas campestris* pv. *pruni* under various incubation conditions[x]

Incubation at (light, temperature)	Sites inoculated on 10 leaves (no.)	Sites (%) infected with inoculum (cfu/ml) at: 10^6	10^5	10^4
Dark, 23 ±2 C	100	93 a[y]	51 ab	6 a
Dark, 28 C	100	99 a	69 a	11 a
16-hr photoperiod, 25 C	100	98 a (60–90)[z]	64 a (6–12)	9 a (1–2)
Lab bench, 23 ±2 C	100	60b	43 b	10 a

[x] The inoculum was infiltrated at each site to produce a 2- to 4-mm-diameter water-soaked area and the leaves were placed on 0.5% water agar.
[y] The numbers in columns followed by same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.
[z] Numbers within parentheses are the number of lesions per site.

TABLE 2. Reaction of detached leaves of field-grown peach cultivars to *Xanthomonas campestris* pv. *pruni* strain XP-1[x]

Cultivar	Reported reactions[y]	Infected leaves (no.)	Infected sites (no.)	Lesions/site (no.)
Elberta	HS (19)	8.3	60.7	15.0
Suncrest	HS (8)	8.0	56.0	17.0
Roza	S (8)	7.7	47.7	15.7
Sunhigh	HS (19)	7.3	49.7	14.3
PO-766	S (8)	7.0	46.7	15.0
Harbrite	–	6.7	50.0	17.0
53340	–	6.7	45.3	15.7
McNeely	HR (8)	6.7	50.7	14.7
Loring	R (19)	5.7	36.0	14.3
Harken	–	5.0	31.0	12.0
Redglobe	R (8)	5.0	31.7	15.3
Red Haven	R (19)	4.7	32.0	13.3
Red Skin	R (19)	4.3	21.3	13.3
Jefferson	R (8)	4.0	21.7	13.3
June Gold	R (8)	4.0	27.3	13.3
Candor	HR (19)	3.7	14.7	13.3
Reliance	HR (8)	2.7	7.7	11.0
Washington	R (8)	2.7	19.3	14.3
Rubired	HR (19)	2.0	11.3	5.7
Clayton	HR (19)	2.0	12.7	8.0
Ranger	HR (8)	1.7	11.3	5.3
Nemaguard	HR (8)	1.7	7.3	3.3
LSD ($P = 0.05$)		1.2	9.8	4.0

[x] Inoculum of XP-1 (10^6 cfu/ml) in sterile distilled water was infiltrated to produce 3- to 4-mm-diameter water-soaking at eight selected sites on each leaf. Nine leaves replicated three times were inoculated.
[y] Field resistance rating previously recorded (8,19). HR = highly resistant, R = resistant, S = susceptible, and HS = highly susceptible.

hand, leaves raised 3 mm above water agar, by supporting them on sterile toothpicks laid flat on the agar, became dry. When kasugamycin (16 and 32 μg/ml) and cycloheximide (200 μg/ml) were incorporated into the water agar, the leaves became chlorotic and senescent. When untreated leaves were placed on water agar, saprophytic fungi (approximately 10 colonies per leaf) and bacteria grew beneath leaves. Excessive contamination caused leaf deterioration. Treatment of leaves with 70% ethanol removed the contaminating bacteria and fungi. Sodium hypochlorite at 1% was also an effective disinfestant but the treated leaves developed white flecks after 4–5 days. Disinfestation of field-collected leaves with ethanol treatment required more time (90 sec) than for greenhouse leaves (60 sec). No visible changes occurred when leaves were incubated at 22–25 C under dark or light conditions. Incubation of leaves at 28 C in continuous dark favored contaminants and resulted in extensive water-soaking of some leaves. Sealing of petri plates with Parafilm to conserve moisture was not necessary.

Leaf response to inoculation. The infiltration technique caused minimum injury to leaves. Tiny yellowish discoloration marks caused by the syringe during inoculation occurred only at few sites after 2–3 days. Distal leaf portions were preferred for inoculation because of the ease of infiltration. The water-soaking caused by inoculum infiltration disappeared in 1–24 hr. All three strains of *X.c.* pv. *pruni* caused similar symptoms. At inoculum concentrations of 1×10^7 cfu/ml or more, all inoculated sites exhibited confluent water-soaking after 6–9 days. These became dark brown and brittle necrotic spots, often surrounded by greyish white or purple margin. The sites showing symptoms could be distinguished 2–3 days earlier when viewed by transmitted light. Bacterial ooze occurred frequently from older lesions. Although inoculum was frequently spilled at random, symptoms developed only at infiltration sites. At inoculum concentrations of 1×10^6 and 1×10^5 cfu/ml, symptoms developed in 9–14 days. Less variation in symptom manifestation occurred between sites on one leaf than on sites on different leaves. Types of symptoms expressed varied from leaf to leaf. In general, these symptoms in descending order of frequency were discrete, water-soaked lesions (Fig. 1); greyish area with blackening of veins; minute greyish white lesions with dark necrotic centers; and slight chlorotic discoloration. Slight chlorotic discoloration was not a specific response and was not considered a pathogenic reaction. Incubation of inoculated leaves under different temperature and light conditions (Table 1) resulted in frequent infection. However, well-defined symptoms developed only on the leaves maintained at 25 C and 16 hr photoperiod with fluorescent lights. Under these conditions, disease lesions were readily enumerated and were directly proportional to inoculum concentration. When the leaves were incubated in the dark, some inoculation sites remained water-soaked throughout the test period. This masked any distinct lesions that might have developed.

Disease incidence on attached and detached leaves had correlation coefficients of 0.80 and 0.79 with respect to number of leaves and individual sites infected, respectively. Qualitative responses of detached and attached halves of the same leaf were generally similar. On two of ten shoots, however, most detached leaf portions showed water-soaked lesions compared to greyish white lesions on attached leaf halves. Some differences in lesion type occurred from plant to plant. Although it was more difficult to infiltrate the bacterial suspension, young attached leaves developed lesions that were more sharply defined. Individual lesions were well separated as the leaves continued to grow and expand. Older leaves showed less distinct symptoms (blackening of veinal network). Detachment of young leaves stopped their growth and expansion, and lesions that developed were confluent. On detached leaves, well defined lesions were expressed on relatively young but fully expanded leaves (e.g., the fourth to sixth leaf from shoot tip).

Inoculation by placing inoculum droplets on leaf surface generally elicited no response on attached or detached leaves at inoculum concentrations as high as 1×10^8 cfu/ml. Occasionally, a few greyish white lesions developed at some sites when the attached leaves were incubated up to 30 days.

Leaf response specificity. Infiltration of bacterial mixtures that contained no detectable *X.c.* pv. *pruni* caused no leaf response. Five of 51 individual epiphytic strains caused a hypersensitive reaction within 24–48 hr at inoculum concentrations of 1×10^6 cfu/ml or more. The inoculated area turned necrotic and tended to fall out of the leaf, giving a shot-hole symptom. Similar reactions were observed on attached leaves. Some strains caused slight chlorotic discoloration of inoculated sites. Leaf response, if any, to saprophytes was apparent in 1–3 days. Symptoms of the types caused by *X.c.* pv. *pruni* were not caused by other bacteria.

Cultivar resistance. The number of leaves and individual sites infected were generally related to the previously reported differential resistance reactions except for cultivar McNeely (Table 2). Four of seven highly resistant cultivars also developed significantly fewer lesions per site. At an inoculum concentration of 1×10^5 cfu/ml, leaves of greenhouse-grown Sunhigh seedlings were more susceptible (100% of the leaves and 93% of the sites infected) than those of field-grown Sunhigh trees (64% of the leaves and 55% of the sites infected). Based upon significant differences in disease incidence at $P = 0.05$ according to test of least square differences (Table 2) the field resistance of peach cultivars can be classified according to detached leaf response (Table 3).

TABLE 3. Prediction of field resistance of peach cultivars to *Xanthomonas campestris* pv. *pruni* based on detached leaf response

Predicted field resistance[z]	Detached-leaf response	
	Leaves infected (%)	Sites infected (%)
Highly resistant	0–30	0–20
Resistant	31–55	21–50
Susceptible	56–85	51–70
Highly susceptible	86–100	71–100

[z] Possible field resistance is predicted on the basis of apparent correlation between resistance previously reported and detached leaf response (Table 2).

DISCUSSION

Detached peach leaves provide a relatively simple and specific method to quantitatively define virulence of *X.c.* pv. *pruni* and plant susceptibility. The excellent keeping quality of detached peach leaves placed on water agar and their specific disease response is remarkably similar to that of attached leaves. The ease of manipulation of leaves has made it possible to reduce variation due to resident saprophytic populations by ethanol disinfestation and make precise inoculations in a controlled environment. Furthermore, the number of lesions produced is directly proportional to inoculum dosage. The infiltration of 70 cfu per site from inoculum of *X.c.* pv. *pruni* containing 1×10^6 cfu/ml and the number of individual defined lesions produced at each site (Table 1) indicate that a single cell is capable of forming a lesion and thus confirming earlier observations (3). The failure of spilled-over but uninjected inoculum to cause symptoms is probably due to ineffective entry of the bacterium in leaf tissue. This confirms earlier observations (5) that windy rains are necessary to cause water congestion and infection of peach leaves. Water congestion is important to produce continuous water columns between the inside and outside of leaves and facilitates bacterial entry through stomata (5). Based on previous experiments (5,11,12) and field observations that greater water-soaking of leaves and infection by *X.c.* pv. *pruni* occur on the side of a tree receiving windy rain, it is assumed that infiltration of inoculum in tissue is essential in achieving increased infection. Furthermore, distal portions of leaves develop more infection in the field and are easily infiltrated in the laboratory. Inoculation of detached leaves by infiltration is, therefore, similar to the natural bacterial infection of peach leaves.

Normally, infiltration of inoculum into a leaf is not recommended (7) possibly because of damage to the mesophyll tissue. Use of a needle-less syringe and leaf support by layers of paper towel during our inoculations, however, resulted in minimal injury. The majority of water-inoculated sites could not be distinguished from the surrounding healthy tissue. Furthermore, use of a needle-less syringe makes it possible to inoculate 25–35

sites per minute and to quantify infectivity with defined inoculum delivery. Detached fruits of peach, plum, apricot, and cherry can also be used to detect fruit susceptibility to *X.c.* pv. *pruni* (7). On the other hand, inoculation of detached bean pods with *X.c.* pv. *pruni* results in nonspecific responses (18).

High concentrations of *X.c.* pv. *pruni* do not clearly distinguish resistance among peach cultivars using detached leaves; however, use of lower concentrations (1×10^5–1×10^6 cfu/ml, a situation more similar to field conditions) permits detection of differential resistance to infection by *X.c.* pv. *pruni*. The comparative susceptibility of detached leaves of different peach cultivars was directly related to susceptibility under field conditions (Table 2). Field resistance of a given cultivar can therefore be predicted based on the number of detached leaves and individual infiltration sites that become infected (Table 3).

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