Quantitative Aspects of Pathogenesis of Xanthomonas pruni in Peach Leaves

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ABSTRACT

An assay system for quantitatively defining the virulence of *Xanthomonas pruni* in its natural host is described. Approximately 10μl of inoculum containing approximately 10^{2} to 10^{4} colony-forming units (CFU)/ml was introduced, by spraying at 1.25×10^{-1} Kg/cm^2, into the intercellular spaces at selected circular areas of young Sunhigh peach seedling leaves. The assay can be completed in 14 days. The number of lesions induced by *X. pruni* in this system is directly proportional to the number of CFU in the inoculum. Approximately 16 to 18 CFU are required to cause a single lesion, indicating that the efficiency of this system is relatively high. Comparative analyses of log-dose/probit-response data of four *X. pruni* isolates are presented. In infectivity titrations of *X. pruni,* quantal (all-or-none) responses are conveniently recorded as the presence or absence of a lesion at an inoculation site. Determination of slope and median effective dose (ED_{50}) values from the dose-response curves allows comparison of the virulence of *X. pruni* isolates and of host susceptibility, and permits conclusions about how the bacterium produces the response. Since the slopes of the dose-response curves are less than two, *X. pruni* cells probably act independently during growth in vivo to cause infection and a single *X. pruni* cell is probably capable of causing infection if the proper environment is available. Based on comparative ED_{50} values, a sweet cherry isolate of *X. pruni* is less virulent on Sunhigh peach seedling leaves than peach, apricot, and plum isolates.

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Virulence of a pathogen is usually determined in an infectivity titration assay from the quantitative relationship between concentration of the infectious agent in the inoculum and the frequency of a specific host response (24). However, studies on the intrinsic nature of woody plant-phytopathogenic bacteria interactions are generally hampered by the lack of a suitable system to evaluate pathogen virulence and host susceptibility. Additionally, definitions of host range, host specificity and virulence alterations of phytopathogenic bacteria are

generally inadequate because information about dose-response relationships in various systems is not available (11, 12). Bioassays based on quantitative and quantal (all-or-none) responses are described for some phytopathogenic bacteria, including *Agrobacterium tumefaciens* (19), *Corynebacterium michiganense* (8, 9, 18), *Erwinia carotovora* var. *atroseptica* (28), *E. carotovora* var. *carotovora* (28) and several *Pseudomonas* species (11, 20) using natural or artificial hosts. In other cases, evaluation of the virulence of phytopathogenic bacteria in their natural hosts is based on various types of disease indices (2, 3, 4, 16), or on the rate of disease system development, such as lesion and canker size (13, 15, 26, 34), following inoculation with high concentrations of inocula containing from 10^6 to 10^8 cells or colony-forming units (CFU)/ml. However, a biological interpretation of the relationship between inoculum dose and intensity of a response in terms of pathogen virulence may not always be possible when high concentrations of inoculum are used (24).

The frequency of responses in infectivity titrations with microorganisms in living hosts is often related to the log of the inoculum dose by an integrated log-normal distribution which forms a normal sigmoid curve (14, 24). Linearity of a log-normal dose-response curve can be achieved by transforming the expected proportion of response values into probits (14), and plotting the probit values against the log of the inoculum dose. In infectivity titrations based on quantal responses which are transformed into probits, the parameters of the log-dose/probit-response curves are the median effective dose (ED_{50}), corresponding to a probit of 5, and the slope (b). The relation between the probit of the expected proportion of responses and the dose is the linear equation (14):

$$Y = a + bX.$$

Due to the small slope values of dose-response curves, the precision of infectivity titrations based on quantal responses is inherently low and measurement of quantitative responses in which each inoculated subject responds may be preferable in some cases (23). Nevertheless, analyses of log-dose/probit-response relationships have been used successfully to study the effect of various factors on the response of plants, and to interpret the etiology of plant responses to various phytopathogenic bacteria (5, 8, 9, 10, 11, 28). Thus, in natural host-phytopathogenic bacteria interactions, inoculated bacterial cells act independently during infection; whereas in artificial plant-bacterial pathogen combinations, the challenging bacterial cells cooperate to induce a response by the host (8, 9, 10, 11, 28).

The purpose of this paper is to describe a system for quantitatively defining the virulence of *Xanthomonas pruni*, and the response of stone fruit hosts to infection by this pathogen. This system may be useful for studying the basic nature and development of infection by *X. pruni*, and for evaluating resistance or susceptibility of stone fruit hosts to *X. pruni*. The application of the hypothesis of independent action (8, 9, 11, 24, 25, 28, 31) to the response of peach to *X. pruni* infection is also described.

MATERIALS AND METHODS.—Isolates of *X. pruni* were obtained from naturally infected leaves of peach and sweet cherry seedlings, and from apricot (cultivar Blenril) and plum (cultivar Burmosa) trees. Cultures were maintained on 2.3% nutrient agar

TABLE 1. Effect of inoculum concentration on *Xanthomonas pruni* pathogenesis in young Sunhigh peach seedling leaves

Approximate inoculum concentration[a] (CFU/ml)	Time between inoculation and initial reaction (days)	Reaction at inoculation site
10^8	2	chlorosis; necrosis, with surrounding greyish-white area; becoming dry and brittle
10^7	2	chlorosis; necrosis, with surrounding greyish-white area
10^6	2	general chlorosis with some surrounding greyish-white area
10^5	2	discrete greyish-white spots (lesions) that enlarge and may coalesce into larger areas; centers of initial lesions become necrotic
10^4	3	many discrete greyish-white spots (lesions) that develop necrotic centers
10^3	6	several discrete greyish-white spots (lesions) that develop necrotic centers
10^2	8	few discrete greyish-white spots (lesions) that develop necrotic centers
10^1	14	no reaction

[a]Log phase cells of *X. pruni* (apricot/Blenril isolate). Plants received no pre-inoculation treatment.

supplemented with 2.0% glucose (NGA) at 27 C and transferred weekly. Titer determinations of all cell suspensions were made on 2.3% nutrient agar with 0.2% glucose and 0.5% NaCl (NGSA).

Inocula were prepared as follows: Log phase cells from 0.8% nutrient broth with 0.2% glucose and 0.5% NaCl (NGSB) shake cultures were collected by centrifugation at approximately 5,000 *g* for 10 to 15 minutes. Centrifuged cells were resuspended in sterile distilled, demineralized water (SDDW). Cell suspensions were adjusted turbidimetrically to contain approximately 1 to 3×10^8 colony-forming units (CFU)/ml (absorbance at 620 nm with a 1.0-cm light path was 0.1 to 0.15) and diluted serially 10-fold in SDDW to the required concentrations. Inocula were used immediately to inoculate plants. To determine dose-response relationships, the inoculum doses, obtained by 2-fold serial dilutions of a stock cell suspension containing approximately 1 to 2×10^4 CFU/ml, were selected to give 1 to 99% expected responses. Responses outside this range correspond to very extreme probits that carry little weight and may be disregarded (14). The titer of each inoculum was determined by spreading 0.1 ml of an appropriate dilution on the surface of NGSA, incubating

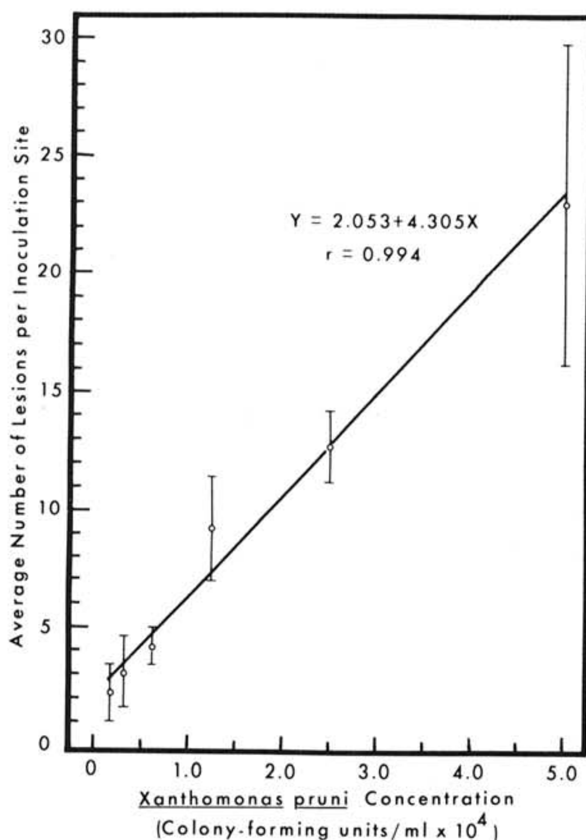

Fig. 1. Proportional relationship between the average number of lesions per inoculation site and the concentration of *Xanthomonas pruni* in the inoculum. Each point is the average number of lesions at six inoculation sites in each of the three experiments. One standard error from each mean is indicated by the brackets.

the plates at 27 C, and counting the colonies two to three days later.

For analyses of dose-response relationships, Sunhigh peach seedlings were held in a controlled environment from (CER) at 27 ± 2 C and near 100% relative humidity for 20-24 hours before inoculation. One to three young terminal leaves on each of two to three shoots per seedlings were inoculated as previously described (27). Inocula were applied to selected, circular areas on the lower leaf surface, and exposed through a hole (5 to 6 mm in diameter) in an aluminum foil shield, by spraying at 1.25×10^{-1} Kg/cm^2 until the underlying tissue was uniformly watersoaked. Excess inoculum was rinsed off with SDDW immediately. After inoculation the seedlings were maintained in the CER for five days before being returned to normal greenhouse conditions. Based on the assumption that 1.0 ml of distilled water weighs 1.0 g, and based on the average weight increase of leaves immediately following uniform infiltration of distilled water into the tissue at selected inoculation sites, approximately 10 μl of inoculum were introduced into the intercellular spaces at each inoculation site.

Quantitative responses were recorded as the number of lesions at each inoculation site. Lesions were counted 14 days after inoculation. By analogy with the tumor-forming unit of *A. tumefaciens* (19) and the lesion-forming unit (LFU) of *P. savastanoi* (20), a LFU for *X. pruni* is defined herein as the number of bacteria (CFU/ml) divided by the mean number of lesions per inoculation site. Thus, the LFU value is an estimate of the number of *X. pruni* cells required to initiate a single lesion.

Quantal responses were recorded as the number of inoculation sites, with at least one lesion (positive) or without any visible lesions (negative) up to 14 days after inoculation. Plant response records were subjected to probit analyses using the maximum likelihood estimation (14) to characterize the parameters of the log-dose/probit response curve, the mean and slope, and to derive the probit regression equation. The computations were simplified by using two computer programs (1, 6).

RESULTS.—*General pattern of X. pruni pathogenesis.*—The effect of inoculum concentration on pathogenesis of an apricot isolate of *X. pruni* in young Sunhigh peach seedling leaves is presented in Table 1. With inocula containing approximately 10^6 to 10^8 CFU/ml, general chlorosis of the entire inoculation site began to develop about two days after inoculation. General necrosis usually developed rapidly within a few days following inoculation with approximately 10^7-10^8 CFU/ml and the inoculation site became dry and brittle. With lower inoculum concentrations (approximately 10^2-10^5 CFU/ml), discrete, greyish-white, circular or angular lesions became visible 2-8 days following inoculation. When the inoculum contained more than approximately 10^5 CFU/ml, the lesions enlarged, and after coalesced into larger greyish-white areas within the inoculation site. The centers of the initial greyish-white lesions eventually became necrotic; and these may have represented the initial infection sites. Eventually the entire lesion became necrotic 14 to 21 days after inoculation. However, the maximum number of lesions that developed at an inoculation site was apparent 10-14 days after

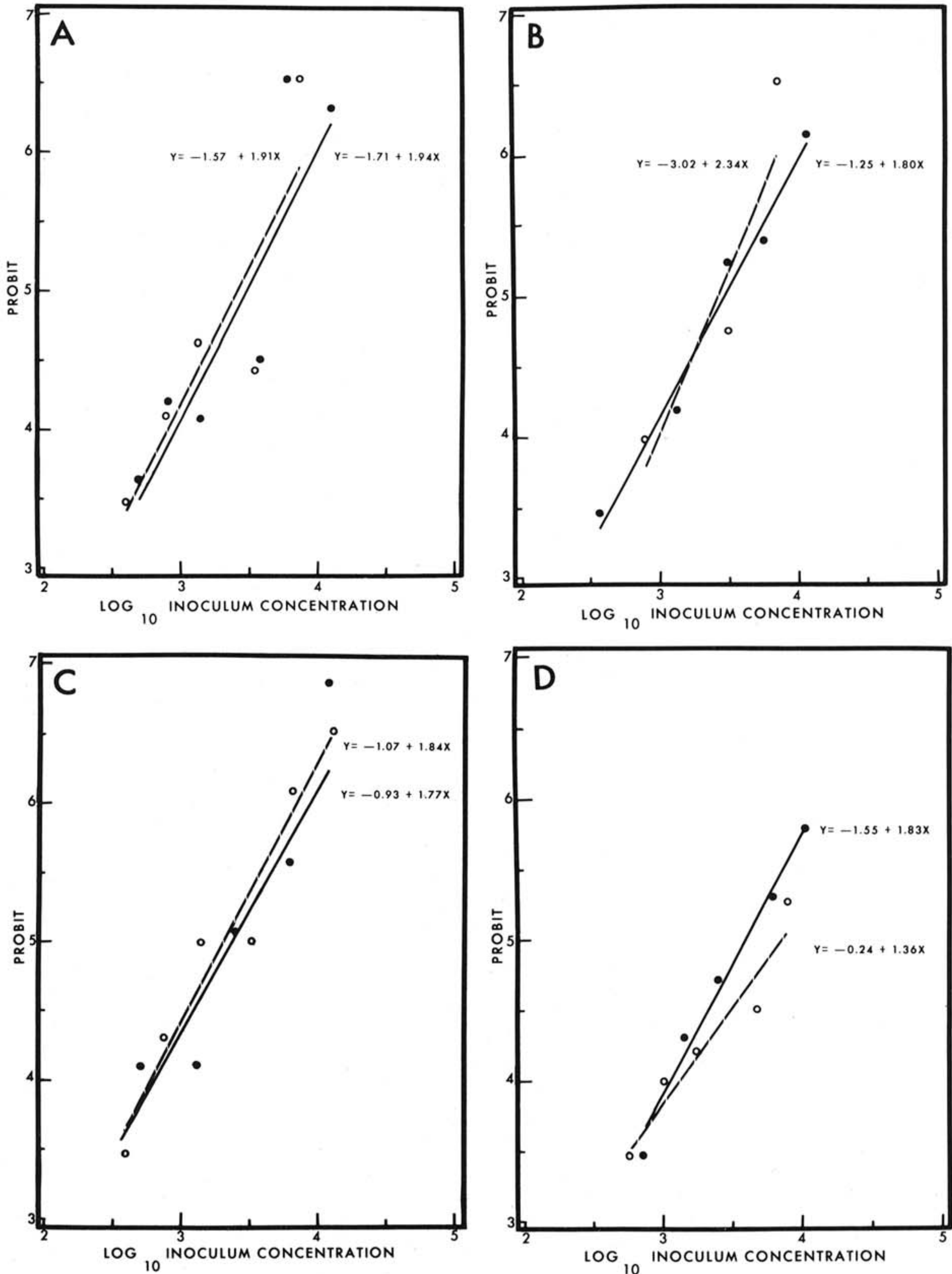

Fig. 2-(A to D). Log-dose/probit-response relationships for **A)** peach, **B)** apricot, **C)** plum, and **D)** sweet cherry isolates of *Xanthomonas pruni* on Sunhigh peach seedling leaves.

TABLE 2. Parameters of the regression curves which describe the log-dose/probit-response relationships for four isolates of *Xanthomonas pruni* on peach cv. Sunhigh seedling leaves

			Probit analysis[a]		Potency probit analysis[b]
X. pruni isolate from	Expt. no.	Number of iterations	Slope (b)	$\log_{10}$ ED_{50}	$\log_{10}$ ED_{50} (95% fiducial limits)
peach	1	4	1.91 ± 0.62	3.44 ± 0.52	3.45 (3.65, 3.25)
	2	4	1.94 ± 0.56	3.47 ± 0.52	3.47 (3.64, 3.30)
apricot	1	4	2.34 ± 0.67	3.42 ± 0.43	3.44 (3.63, 3.24)
	2	2	1.80 ± 0.23	3.47 ± 0.56	3.47 (3.64, 3.30)
plum	1	3	1.84 ± 0.32	3.30 ± 0.54	3.30 (3.54, 3.05)
	2	4	1.77 ± 0.22	3.36 ± 0.56	3.35 (3.53, 3.18)
sweet cherry	1	2	1.36 ± 0.29	3.84 ± 0.73	3.74 (3.99, 3.51)
	2	3	1.83 ± 0.28	3.59 ± 0.54	3.59 (3.77, 3.40)

[a]Probit analysis programs of the Statistical Analysis System and Daum (6).
[b]Daum (6) Potency Probit Analysis program of the data for the eight infectivity titrations. Common slope (regression coefficient) = 1.83 ± 0.14.

inoculation. The size of the lesions ranged from pinpoint to approximately 2 mm in diameter, and may be dependent on the age of the leaf when inoculated. In general, the younger the leaf at the time of inoculation, the larger the final lesion size. Therefore, for all subsequent inoculations, only the youngest terminal leaf on a shoot that would easily accommodate four inoculation sites, 5-6 mm in diameter, was used. However, lesion-size and appearance may also be characteristics of a particular *X. pruni* isolate. In this regard, the sweet cherry isolate consistently induced minute lesions compared to the other three isolates. Alternatively, lesion appearance may be related to the specific *X. pruni* isolate – individual host interaction.

The time interval between inoculation and appearance of lesions induced by *X. pruni* was dependent on inoculum concentration (Table 1). However, the time interval could be reduced, following inoculation with approximately 10^2-10^3 CFU/ml, from five days to three days if the seedlings were held in the CER for 20-24 hours before inoculation. Additionally, the percentage of inoculation sites at which lesions develop was generally increased by this pre-inoculation treatment. This preinoculation treatment was probably related to the efficiency of the water-soaking during inoculation and/or the susceptibility of the tissue. This preinoculation treatment, however, did not affect the type and sequence of lesion development.

Infectivity titration.—The proportional relationship between the concentration of cells of the apricot isolate of *X. pruni* in the inoculum, and the number of lesions at an inoculation site, is presented in Fig. 1. Lesions induced by *X. pruni* at inoculation sites with inocula containing more than approximately 10^5 CFU/ml were too numerous to count accurately. Presumably, most or all of the available infection sites were occupied at these concentrations. Therefore, little change in lesion numbers per inoculation site can be expected with more than approximately 10^5 CFU/ml. However, the number of lesions per inoculation site induced by inocula containing approximately 0.1 to 5 × 10^4 CFU/ml was directly proportional to the concentration of *X. pruni* cells. Thus, an *X. pruni* inoculum containing up to approximately 10^5 CFU/ml, resulting in 1 to 100 lesions per inoculation site, was within the linear portion of the titration curve and was useful for comparing the infectivity of different *X. pruni* isolates.

Based on the assumption that each inoculation site received approximately 10 μl of inoculum, LFU values calculated from the data for the average number of lesions per inoculation site in Fig. 1 ranged from 9 to 23, with an average of 16. Similarly, from the mean inoculum concentration (1.64 × 10^4 CFU/ml) and the average number of lesions per inoculation site (9.07) in Fig. 1, the calculated LFU value is 18. Therefore, an estimated 16 to 18 *X. pruni* CFU are apparently required to induce a single lesion in Sunhigh peach seedling leaves. Although several *X. pruni* cells at each intercellular infection site are apparently necessary to produce a lesion, it cannot be determined from these data whether the inoculated *X. pruni* cells cooperate or act independently during growth in situ to induce a single lesion, provided the proper environment is available.

Log-dose/probit-response relationships.—The log-dose/probit-response relationships based on quantal responses of four *X. pruni* isolates on Sunhigh peach seedling leaves are presented in Fig. 2. The response of each inoculation site based on the presence or absence of at least one lesion was quite variable. The inherently low precision of infectivity titrations based on quantal responses has been discussed elsewhere (24).

Chi-square tests on the infectivity titration data for the peach ($\chi^2_{(3)} = 14.322$ and $\chi^2_{(4)} = 21.269$) and apricot ($\chi^2_{(2)} = 6.323$ and $\chi^2_{(4)} = 2.027$) isolates indicated heterogeneity of departure of the observed values from the probit regression lines. Nevertheless, there was no clear indication of systematic deviation from linear regression in any of the eight infectivity titrations. Chi-square tests on the data from the infectivity titrations of the plum ($\chi^2_{(4)} = 2.475$ and ($\chi^2_{(5)} = 5.721$) and sweet cherry ($\chi^2_{(3)} = 2.142$ and $\chi^2_{(3)} = 0.657$) isolates gave no evidence for heterogeneity of discrepancies between the observed and expected values.

The parameters of the regression curves that describe the log-dose/probit-response relationships for the four *X. pruni* isolates are presented in Table 2. The probit slope (regression coefficient) values in the infectivity titrations were less than two, ranging from 1.36 to 1.94, in all but

one case. In one infectivity titration of the apricot isolate, the slope value was 2.34. Thus, *X. pruni* cells probably act independently during growth in vivo to cause a lesion to develop in susceptible *Prunus* species leaf tissue, since the probit slopes are less, or not significantly greater, than two (11, 24, 28, 29). The $\log_{10}$ ED_{50} values for these four *X. pruni* isolates ranged from 3.30 to 3.84, and are of the same order of magnitude as the corresponding values for *P. morsprunorum* on cherry and *P. syringae* on pear (11).

A test for parallelism of the regression lines in the Potency Probit Analysis (6) of the data from the eight individual infectivity titrations gave a chi-square value, with 7 degrees of freedom, of 4.796, indicating the slope values are similar. In the Potency Probit Analysis (6), the new approximation of the common slope for the eight infectivity titrations was 1.83 ± 0.14. This also supports the conclusion that *X. pruni* cells probably act independently during growth in vivo to cause infection in this system.

The $\log_{10}$ ED_{50} values and their 95% fiducial limits for the four *X. pruni* isolates of Sunhigh peach seedling leaves are also presented in Table 2. The corresponding ED_{50} values (CFU/ml) and their 95% fiducial limits are: 2,818 (4,467, 1,778) and 2,951 (4,365, 1,995) for the peach isolate; 2,754 (4,266, 1,738) and 2,951 (4,365, 1,999) for the apricot isolate; 1,995 (3,465, 1,122) and 2,239 (3,388, 1,514) for the plum isolate; and 5,495 (9,772, 3,236) and 3,890 (5,888, 2,512) for the sweet cherry isolate. Based on the average ED_{50} value from two infectivity titrations for each *X. pruni* isolate on Sunhigh peach seedling leaves, these isolates can tentatively be rated in decreasing order of virulence as: plum (average ED_{50} = 2,117 CFU/ml), apricot (average ED_{50} = 2,852 CFU/ml), peach (average ED_{50} = 2,884 CFU/ml), sweet cherry (average ED_{50} = 4,692 CFU/ml). The sweet cherry isolate was consistently less virulent than the other three isolates, since more than twice as many CFU were required to cause at least one lesion at 50% of the inoculation sites as for the plum isolate. The plum isolate had the lowest ED_{50} values in both infectivity titrations.

In the Potency Probit Analysis (6, 14), improved estimates of the regression curves are based on a new approximation of the common slope. This probably accounts for the slight differences in the $\log_{10}$ ED_{50} values between those obtained from the Probit Analysis of each infectivity titration individually, and those obtained for each *X. pruni* isolate from the Potency Probit Analysis.

DISCUSSION.—Analyses of the interactions between phytopathogenic bacteria and their hosts require a relatively specific method with which bacterial virulence and plant susceptibility can be quantitatively defined. Such a method should also be useful for defining host range and host specificity, especially among phytopathogenic xanthomonads.

Infectivity titrations can be based on quantitative and quantal responses (5, 12, 24). Quantal responses, which can be only one of two alternatives, are usually expressed as the proportion of inoculated subjects that respond at various inoculum doses, and are most precisely estimated by the dose (ED_{50}) which causes 50% of the subjects to respond (5, 8, 9, 11, 12, 24, 28). Quantitative responses can take any one of a series of values and can be measured at inoculum doses that cause all inoculated subjects to respond (5, 24). Various types of quantitative responses measured in infectivity titrations of phytopathogenic bacteria in woody plant hosts include lesion number, lesion size, canker size, and response time (10, 15, 24, 34).

Due to apparent host specificity, use of artificial hosts for quantitative studies of the infectivity of most phytopathogenic *Xanthomonas* species, including *X. pruni*, may not be suitable. Furthermore, there may or may not be any specific pathological relationship between a response on an artificial host and virulence of the pathogen on its natural host. Finally, responses such as local necrotic lesions or hypersensitive reaction on artificial hosts usually require large numbers of incompatible bacteria (17, 18, 20, 22, 32). Unequivocal evidence for qualitative and quantitative changes in host specificity or virulence of phytopathogenic *Xanthomonas* species using artificial hosts is not available (7, 21, 30, 32). This may be due, at least in part, to the lack of a suitable quantitative method with which to interpret the response of different host-pathogen systems. In fact, the relative infectivities of several *Pseudomonas* spp. in natural and artificial hosts could not be obtained by direct comparison of respective ED_{50} values, because of large differences in the corresponding slope values (11). Nevertheless, relative infectivity differences in natural and artificial host-bacteria concentrations may be more marked with low doses of inoculum (11). The number of dead plant cells can be detected in otherwise symptomless tobacco leaves inoculated with concentrations of the incompatible pathogen, *P. pisi*, lower than those required to give a confluent hypersensitive reaction, and is directly related to the number of *P. pisi* cells in the inoculum (33). In contrast, the ratio of bacteria cells to dead plant cells in the incompatible interaction between *E. amylovora* and tobacco leaves was 10:1 (33). While this phenomenon may exhibit certain characteristics of a confluent hypersensitive reaction induced in various incompatible host-pathogen combinations, plant leaf cell death may or may not be caused by bacteria acting independently in situ.

Young leaves of Sunhigh peach seedlings are suitable for a relatively sensitive and specific assay for quantitatively defining the infectivity of *X. pruni*. Thus, *X. pruni* virulence can be expressed in terms of LFU and/or ED_{50} of a particular isolate. The assay can be completed 10-14 days after inoculation. Use of computer programs simplifies probit analysis calculations of the response data.

With inocula containing less than approximately 10^5 CFU/ml, there is a linear relationship between the number of lesions which develop at inoculation sites and the concentration of *X. pruni* cells in the inoculum. The LFU values reported herein for *X. pruni* on Sunhigh peach seedling leaves are considerably lower than similar values calculated from the data presented for another natural bacterium-host combination, *C. michiganense* on tomato (18). However, cells of *C. michiganense* apparently do act independently during infection of tomato plants (8, 9).

The hypothesis of independent action (24) for *X. pruni* during infection of Sunhigh peach seedling leaves was established by analyses of log-dose/probit-response data from infectivity titrations based on quantal responses.

Thus, the probit slopes for infectivity of four different isolates of *X. pruni* were similar and not significantly greater than two (8, 9, 11, 24, 25, 28, 29). Furthermore, the time for lesion development appeared to be the same with inocula containing less than 1 ED_{50}. Presumably, this is due to cells in the inocula acting independently in vivo (24). These results confirm previous reports that a single cell of a phytopathogenic bacterium is capable of infecting its natural host (8, 9, 11, 28), and extend this observation to a phytopathogenic *Xanthomonas* sp.

Comparison of LFU and ED_{50} values from infectivity titrations for various *X. pruni* isolates on their usual *Prunus* species hosts might reveal differences in virulence, and provide quantitative means for studying the infection process. Susceptibility of various *Prunus* species may also be quantitatively defined by LFU and ED_{50} values. Alternatively, standardized dose-response time relationships (10, 24) might be established to quantitatively define resistance to *X. pruni* infection.

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