Comparison of Resistance in Tobacco to *Pseudomonas syringae* pv. *tabaci* Races 0 and 1 by Infectivity Titrations and Bacterial Multiplication

K. K. Knoche, M. K. Clayton, and R. W. Fulton

First and third authors, Department of Plant Pathology, and second author, Department of Statistics and Department of Plant Pathology, University of Wisconsin-Madison 53706.

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ABSTRACT

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The responses of tobaccos susceptible to *Pseudomonas syringae* pv. *tabaci* race 0 and 1, susceptible only to race 1, and susceptible to neither race were compared by infectivity titrations. When quantal responses were recorded, values of median effective dose and slope of the log-dose/logit-response curve were used to compare resistance. Host-race combinations that were compatible or incompatible in the field behaved similarly in infectivity titration experiments, except for line 8A2S4-9 (resistant to both races), which appeared more susceptible to race 1 than to race 0. The slopes of the response curves indicated that the bacterial cells apparently acted independently in vivo to cause infection, except for race 0 on 8A2S4-9. Lesion development and time to response in incompatible combinations were typical of the hypersensitive response. When bacterial multiplication was measured by infiltrating tobacco leaves with *P. s.* pv. *tabaci* and determining the subsequent changes in population by dilution plating, multiplication was higher in compatible than in incompatible combinations.

Wildfire disease of tobacco (*Nicotiana tabacum* L.), a leaf spot disease, is caused by *Pseudomonas syringae* pv. *tabaci* (Wolf & Foster) Young et al. To control wildfire disease, Clayton (3) developed the resistant breeding line TL106 from a backcross of *N. tabacum* with the F_1 of a *N. longiflora* Cav. and *N. tabacum* cross. Cultivars containing TL106-derived resistance, Havana 501 and an improved cultivar, Havana 503, were released in Wisconsin in 1963 and 1965, respectively (12,19). Skoog and Fulton (21) reported a strain of the wildfire-causing bacterium infecting these and other cultivars of TL106 parentage. The original wildfire-causing strain that is avirulent on TL106-derived tobacco was designated as race 0, and the strain virulent on TL106-derived tobacco was designated as race 1 (22). Resistance to race 1 was transferred from *N. rustica* L. to *N. tabacum* breeding lines by Stavely and Skoog (23,24).

Although resistance in TL106 and a cultivar susceptible to both races of *P. s.* pv. *tabaci* have been compared by measuring multiplication of bacteria in vivo (5), infectivity titrations have not been used. Infectivity titrations, which examine the relationship between the concentration of bacteria and frequency of a specific host response (8), have typically been used to evaluate host resistance and pathogen virulence and to study the etiology of plant responses in other plant-bacterial pathogen interactions. In infectivity titrations, quantal (all-or-none) response curves are linearized, often using probit analysis, and the median effective dose (ED_{50}) and slope of the dose response curve are calculated (10,17). The slope is used to assess whether the challenging bacteria act independently or cooperatively in causing the plant response (7,18,20).

Probits are not the only transformations that may be used: logits, as well as other transformations, will linearize the dose-response curve (1,4,10). As in probit analysis, logistic analysis provides estimates of the ED_{50} and slope. In fact, both logit and probit transformations are quite similar (4) and only slight, if any, differences in ED_{50} estimates will occur when estimated by logistic and probit analysis (1).

The purpose of this study was to compare reactions of Havana 142, Havana 503, and line 8A2S4-9 to *P. s.* pv. *tabaci* races 0 and 1 by logistic analysis of infectivity titrations and multiplication of bacteria in vivo. In the field, Havana 142 is not resistant to either race, Havana 503 is resistant only to race 0 and was derived from TL106, and 8A2S4-9 is resistant to both races 0 and 1 with resistance to race 1 derived from *N. rustica*, and the source of resistance to race 0 may be derived from either *N. rustica*, *N. longiflora*, or both. A preliminary report of portions of this work has been presented (16).

MATERIALS AND METHODS

Plants and bacterial cultures. *P. s.* pv. *tabaci* race 0 (ATCC11528) was obtained from R. D. Durbin, University of Wisconsin-Madison. *P. s.* pv. *tabaci* race 1 (Vir 78) was isolated from diseased tobacco in Wisconsin. Cultures were stored in nutrient broth at 4 C. Bacteria were grown for 48 hr at 28 C on King's B agar (14).

N. tabacum cultivars Havana 142 and Havana 503 are commercial tobacco varieties, and breeding line 8A2S4-9 was developed by R. W. Fulton. For infectivity titrations, tobacco was grown in a growth chamber at 28 C with a relative humidity of 50–70% and a 12-hr photoperiod (275–300 $\mu m^{-2} s^{-1}$), provided by fluorescent tubes and incandescent bulbs. Seed was sown in 4-in. clay pots in steamed muck soil. Two to three weeks after germination, seedlings were transplanted into 6-in. plastic pots, then watered and fertilized daily with Hoagland's solution (13). For bacterial multiplication studies, plants were cultivated as indicated above except they were grown in a greenhouse in a steamed mixture of three parts soil and one part sand.

Breeding line 8A2S4-9 was the result of a series of crosses and backcrosses of a line derived from *N. rustica* that was resistant to race 1. Some crosses were with lines carrying resistance to race 0 derived from *N. longiflora*, whereas other crosses were with tobacco susceptible to both races but having desirable agronomic characters. Resistance to race 0 in Havana 503 is inherited as a single dominant factor (22). The resistance to races 0 and 1 in 8A2S4-9 in subsequent selfed generations was inherited erratically, suggesting that the resistance to race 0 was derived from *N. rustica* rather than *N. longiflora*.

Inoculation of plants. In all experiments, plants were inoculated by injecting bacterial suspensions into the leaf mesophyll with a

hypodermic syringe and 28-gauge needle 1 mo after transplanting. The infiltrated area was then outlined with a water-resistant felt tip marker to mark the inoculation site.

Infectivity titrations. To prepare inoculum for infectivity titrations, bacteria were suspended in sterile distilled water, washed once, and adjusted to $A_{600nm} = 0.1$. Serial dilutions were made in sterile distilled water. The concentration of viable bacteria at each inoculum level was determined by dilution plating.

For infectivity titrations involving response time and symptoms, a fivefold dilution series of *P. s.* pv. *tabaci* races 0 and 1 was made. Twelve inoculum concentrations, ranging from 2.08×10^8 to 6.70×10^{-1} cfu/ml, for each race were chosen and inoculated onto four plants of each cultivar or breeding line. On each plant, all 12 inoculum levels were infiltrated into two adjacent leaves. The placement of the inoculum levels on the leaves was randomized. Response time and symptoms were recorded daily for 14 days. Two infectivity titrations were done in this manner.

For infectivity titrations involving quantal responses, a threefold dilution series of *P. s.* pv. *tabaci* races 0 and 1 was made. Inoculum levels of *P. s.* pv. *tabaci* ranging from 4.5×10^7 to 1×10^0 cfu/ml for race 0 and 3.1×10^7 to 1×10^0 cfu/ml for race 1 were chosen from the dilution series to inoculate 12 plants of each cultivar or breeding line. On each plant, 13 dilutions of race 0 were inoculated onto one leaf, and 12 dilutions of race 1 were inoculated onto an adjacent leaf. The locations of the dilutions were randomized on each leaf. Eight days after inoculation, symptoms were recorded. Every infiltrated area was recorded as either responding, i.e., necrosis developed, or not responding, i.e., necrosis did not develop. Quantal infectivity titrations were replicated three times.

Dose-response data for each quantal response experiment were analyzed using the BMDP Stepwise Logistic Regression Program (6). In the analysis, the data for each host-race combination were fitted by the maximum likelihood method to a logistic model with the following form:

$$\text{logit}(1 - S) = \alpha + \beta x,$$

where $1 - S$ is the probability of a plant responding at dose x, x is the log concentration of bacteria (cfu/ml) in the dilution, α is the intercept of the model and β is the slope of the model. Goodness-of-fit chi-square and improvement chi-square tests were used to test the fits of the models and to judge the appropriateness of condensing data from the three replications (2,6,9). From each model the log ED_{50} and fiducial intervals were computed. Tests were then conducted to compare slopes and intercepts of the models for the different host-race combinations by a stepwise selection procedure based on the improvement chi-square statistic (6,9).

As indicated by Peto (20), under the hypothesis of independent action, a slope in the probit analysis should be approximately 2.0003. A similar approach to calculate the slope for the hypothesis of independent action for logistic analysis was used as follows:

If we set: $y = \text{logit}(1 - S) = \ln[(1 - S)/S]$, we can determine $dy/d(\log n)$ where n is the concentration of bacteria. As in Peto (20),

$$dy/d\log n = dy/dS \times dS/dn \times dn/d\log n.$$

For the logit transformation, $dy/dS = -1/(S[1 - S])$. Using the expressions for dS/dn and $dn/d(\log n)$ given by Peto (20), we obtain

$$dy/d\log n = (-\ln S)(\ln 10)/(1 - S).$$

At the ED_{50}, $S = 0.5$, so $dy/d(\log n) = 2 \times [-\ln(0.5)](\ln 10) = 3.19$. Following Peto (20), we can establish that for independent action the slope of the log-dose/logit-response model should be 3.19 and for cooperative action the slope should be greater than 3.19.

Bacterial multiplication. To prepare inoculum for in vivo multiplication studies, *P. s.* pv. *tabaci* races 0 and 1 were suspended in sterile distilled water, adjusted to $A_{600nm} = 0.01$ and diluted 100-fold. Race 0 bacteria were injected into 20 different sites on one leaf on each of four plants of every cultivar and breeding line. This was repeated with race 1 bacteria with a separate set of plants.

The populations of bacteria in the infiltrated areas were measured at daily intervals for 8 days by removing two 8-mm disks with a sterile cork borer from two randomly chosen inoculated areas on each leaf. Both disks were ground in 2 ml of sterile distilled water in a handheld glass tissue homogenizer. The homogenizer was rinsed twice with 2 ml of sterile distilled water; the 4 ml of rinse water was combined with the homogenate, which was allowed to stand for 5–10 min, then mixed with a Vortex homogenizer and dilution-plated onto King's B agar. The mean colony-forming units per disk and the 95% confidence intervals (25) were calculated for the host-race combination for each day.

RESULTS

Infectivity titrations involving response time and symptoms. Symptom development differed for the various host-race combinations. With host-race combinations Havana 142/race 0, Havana 142/race 1, and Havana 503/race 1 at inoculum concentrations of 10^6–10^7 cfu/ml, the leaf tissue collapsed and became thin and translucent within 1 day. In 1 or 2 days the entire area became necrotic, and, later, necrosis spread and a chlorotic halo developed. At concentrations of 10^4–10^6 cfu/ml the leaf tissue collapsed, became thin and translucent after 2 or 3 days, the entire infiltrated area became necrotic, and a chlorotic halo developed. At concentrations of 10^1–10^4 cfu/ml, small, discrete necrotic lesions (1–3 mm in diameter) appeared after 4 days. Each lesion developed a chlorotic halo.

The hypersensitive response (HR) was found in both Havana 503/race 0 and 8A2S4-9/race 0. At concentrations of 10^6 cfu/ml or above, the infiltrated area became necrotic in 1 or 2 days. Then the lesions bleached to a whitish-tan color, and only a slight halo was seen in some cases. At concentrations below 10^6 cfu/ml no symptoms were evident.

In 8A2S4-9/race 1 at concentrations of 10^6 cfu/ml or above, the tissue collapsed in 1–3 days, and either the entire infiltrated area developed a spreading necrosis and a chlorotic halo, or the entire area became necrotic and bleached to a whitish-tan color. At concentrations of 10^5–10^6 cfu/ml, similar symptoms developed as at higher concentrations, but the tissue collapsed 4 days after inoculation. At concentrations of 10^3–10^5 cfu/ml, discrete necrotic lesions (1–3 mm in diameter) with a chlorotic halo appeared 4 days or later after inoculation.

The interval between inoculation and lesion appearance was negatively correlated with inoculum concentration, decreasing as bacterial concentration increased (Fig. 1). There was a minimum concentration of bacteria below which lesions did not develop. The response time also depended on host-race combination. In Havana 503/race 0 and 8A2S4-9/race 0 combinations, plants responded quickly only at high concentrations. In Havana 142/race 0 and race 1, and Havana 503/race 1 combinations the response time was longer and occurred at lower concentrations. Although 8A2S4-9 is known to be resistant to race 1 in the field, in the growth chamber plants responded at lower concentrations and were more susceptible to race 1 than to race 0.

Infectivity titrations involving quantal responses. Log-dose/logit-response models fitted for each host-race combination for all three replications had p-values of 0.17–1.00 for the chi-square goodness-of-fit test. Improvement chi-square and goodness-of-fit chi-square tests indicated that the data for the three replications could be combined for each host-race combination.

One out of the three replications of the Havana 503/race 0 combination was excluded because the proportion of plants responding to every dilution was only 0 or 1.0, and it is necessary to have responses strictly between 0 and 1.0 for at least two doses to fit the model.

The log ED_{50}'s and slopes of the log-dose/logit-response models for the combined data are shown in Table 1. The small ED_{50} (2.81–3.44) values that were observed for Havana 142/race 0,

Havana 142/race 1, and Havana 503/race 1 indicate that these are compatible combinations. Larger ED_{50} (4.58–6.62) values for Havana 503/race 0, 8A2S4-9/race 0, and 8A2S4-9/race 1 indicate these combinations are incompatible. However, 8A2S4-9 appeared to be more resistant to race 0 than to race 1 on the basis of ED_{50} values. The slopes and 95% confidence intervals for the slopes of the models (Table 1) suggest that the bacteria acted independently except for the 8A2S4-9/race 0 combination. In the 8A2S4-9 combination the slope was significantly greater than 3.19, indicating that the bacteria may have acted cooperatively. Based on the improvement chi-square statistics for the stepwise selection procedure, the model parameters (slope and intercept) were significantly different among the models.

Bacterial multiplication. Both race 0 and race 1 on Havana 142, Havana 503, and 8A2S4-9 multiplied logarithmically, then leveled off and sometimes decreased slightly (Fig. 2). No symptoms developed in incompatible combinations, 8A2S4-9/race 0, 8A2S4-9/race 1, and Havana 503/race 0 (Fig. 2), and bacterial populations multiplied to 10^4–10^6 cfu per leaf disk. In compatible combinations, Havana 142/race 0, Havana 142/race 1, and Havana 503/race 1, tissue collapse began on day 2, which corresponded to the end of the log phase, and necrosis developed

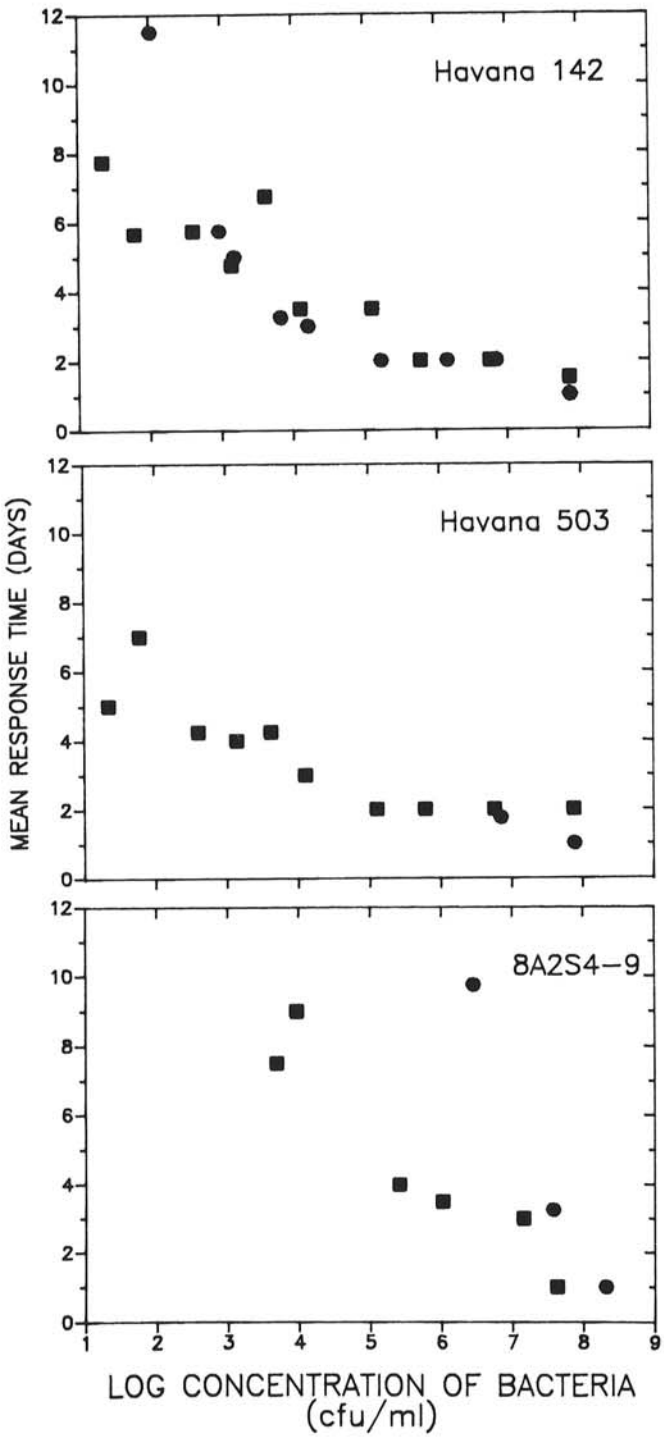

Fig. 1. The relationship between the bacterial concentration (colony-forming units per milliliter) and mean response time of Havana 142, Havana 503, and 8A2S4-9 inoculated with *Pseudomonas syringae* pv. *tabaci* race 0 (•) and race 1 (■). Mean response time is the average response time for four plants at each concentration.

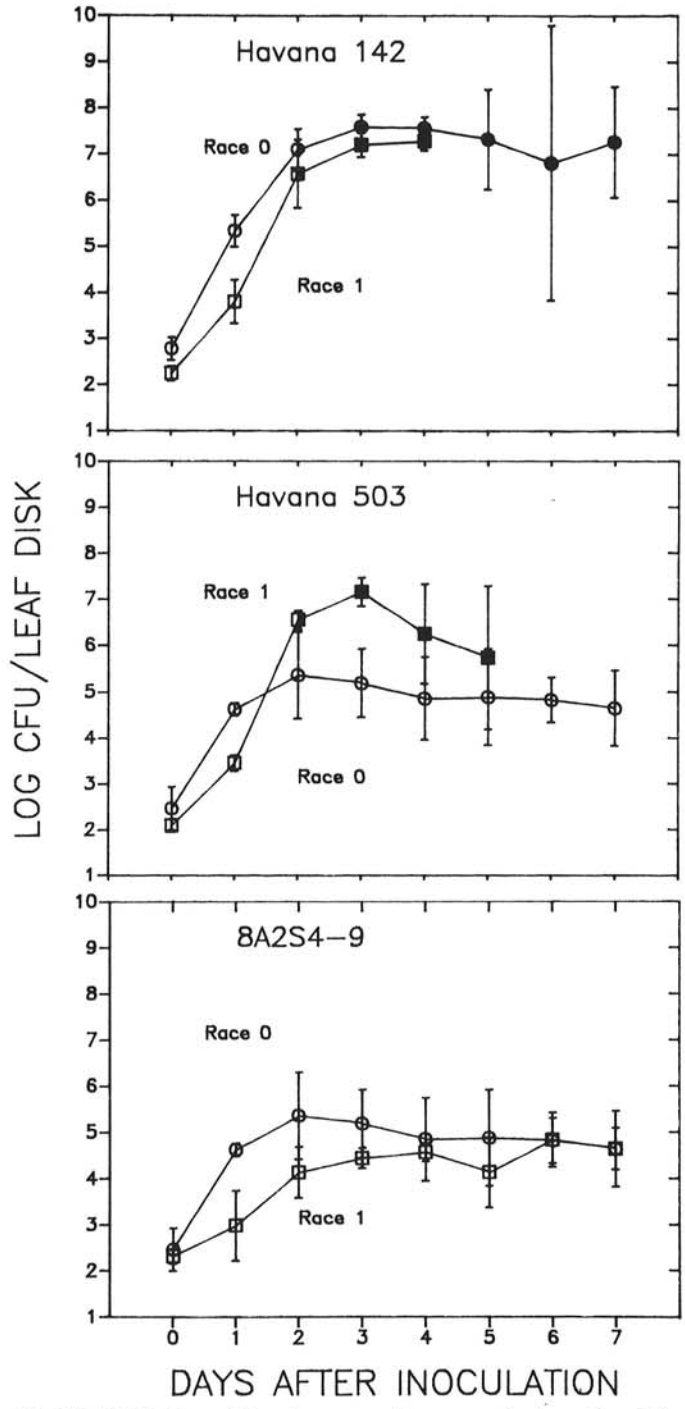

Fig. 2. Multiplication of *Pseudomonas syringae* pv. *tabaci* races 0 and 1 in Havana 142, Havana 503, and 8A2S4-9. Each point is the average $\log_{10}$ colony-forming units per leaf disk (0.50 cm^2) of samples from four plants. A sample consisted of two pooled disks. Error bars represent the 95% confidence intervals (25). Open symbols indicate symptomless tissue, half-closed symbols indicate first appearance of collapse of tissue and closed symbols indicate necrosis of tissue.

by the third day (Fig. 2). The bacteria in these combinations multiplied to populations of 10^6–10^8 cfu per leaf disk. Some data in Figure 2 for race 1 were not included on days 5, 6, and 7 because populations on these days were extremely low after plants were accidentally exposed to low temperatures in the greenhouse.

DISCUSSION

Our results indicate that differences in resistance of tobacco to the races of *P. s.* pv. *tabaci* are reflected both in the ED_{50} values and in differences in bacterial multiplication. Multiplication of the bacteria in tobacco occurred in both compatible and incompatible combinations, but compatible combinations could be distinguished because multiplication was highest in the susceptible tobacco and symptoms were apparent by the third day only in compatible combinations. Diachun and Troutman (5) obtained similar results in multiplication studies with *P. s.* pv. *tabaci* race 0 on susceptible and resistant tobaccos.

A more sensitive measure of differences in resistance of tobacco to *P. s.* pv. *tabaci* races 0 and 1 was obtained by infectivity titrations. From the ED_{50} values it appears that field-resistant 8A2S4-9 is more susceptible to race 1 than to race 0, a difference that was not evident in the bacterial multiplication in vivo experiments.

The slope of the log-dose/logit-response models in the compatible combinations suggests that the bacteria act independently. These results confirm previous reports (7,8,11) that bacteria inoculated into their natural host apparently act independently. In heterogeneous hosts in which an HR occurs, it has been reported that the bacteria apparently act cooperatively (7). In incompatible combinations in which the HR occurs, such as in the 8A2S4-9/race 0 combination, the bacteria appear to be acting cooperatively but in the other incompatible combinations the bacteria appear to be acting independently. We expect that mixed inoculation experiments (7) would clarify how these bacteria infect these cultivars and breeding line.

Logistic analysis was chosen to analyze our infectivity titration data based on the advantages of logits over probits discussed by Berkson (1). Statistically, logistic analysis is superior to probit analysis because logistic estimates are "sufficient" (1): that is, logistic estimates include all relevant information from the observations even with small sample sizes (11). Secondly, Berkson (1) stresses that probit analysis is best justified when the physical properties of the interaction are fully understood. Because this is not the case in the current study, arguments for using probit analysis seem less relevant.

The chi-square tests of fit of the log-dose/logit-response models from infectivity titrations were based on asymptotic chi-square tests, which means that the test statistics do not follow exactly a chi-square distribution but approaches this distribution as the number of observations approaches infinity. This implies that the *p*-values, as well as confidence and fiducial intervals in Table 1, are only approximate. This should be kept in mind when interpreting these results.

Differences in resistance were related to response time and symptom expression in infectivity titrations. In compatible combinations, response time was longer, plants responded at lower concentrations and typical wildfire-disease symptoms were seen. In incompatible combinations response time was short, plants responded only at high concentrations and HR characteristics like those described by Klement et al (15) were evident. Again, it appeared in response time data that field-resistant 8A2S4-9 was more susceptible to race 1 than to race 0. Also, in the incompatible 8A2S4-9/race 1 combination the HR was seen in some infiltrated areas, while other infiltrated areas on the same leaf had typical susceptible reactions.

It is clear from ED_{50} values and response time data that 8A2S4-9 is more susceptible to race 1 than race 0. It is not known whether the resistance to race 0 in 8A2S4-9 is from *N. longiflora, N. rustica*, or both, although we suspect it may be from *N. rustica*. However, the resistances of *N. longiflora* to race 0 and *N. rustica* to race 1 are single dominant genes (22,24), but it is unknown if *N. rustica* resistance to race 0 is a single dominant gene. The different levels of resistance in 8A2S4-9 for each race could be explained by different single dominant genes that control resistance to each race and confer different levels of resistance. Differences in environmental conditions and inoculation procedures in the field and growth room could explain why the difference in resistance was not seen in the field. Differences in symptoms seen on the 8A2S4-9/race 1 combination could be due to variability in the virulence of the bacterium or position on leaf; the leaf tissue with different physiological states could have different levels of resistance.

Bacterial multiplication, ED_{50} values, and response time data reflected differences in the resistance of Havana 142, Havana 503, and 8A2S4-9. However, data from infectivity titrations were more sensitive to differences in resistance than bacterial multiplication measurements.

TABLE 1. Parameters of the logistic models[a] that describe the log-dose/logit-response relationships for three cultivars of tobacco inoculated with the two races of *Pseudomonas syringae* pv. *tabaci*

Cultivar	Race	Compatibility[b]	Log ED_{50}	Log ED_{50} 95% Fiducial Intervals[c]	Slope	95% Confidence Intervals for Slope[d]
Havana 142	0	+	3.44	(3.30, 3.57)	3.66	(2.76, 4.55)
	1	+	3.16	(3.04, 3.27)	2.73	(2.13, 3.34)
Havana 503	0[e]	−	6.30	(6.09, 6.52)	2.65	(1.83, 3.47)
	1	+	2.81	(2.66, 2.94)	2.61	(1.98, 3.23)
8A2S4-9	0	−	6.32	(6.25, 6.39)	5.95	(4.11, 7.79)
	1	−	4.58	(3.94, 5.54)	2.45	(1.97, 2.92)

[a] Logistic analysis from BMDP Stepwise Logistic Regression Program (6). Each model represents data from three infectivity titrations. Twelve plants were used for each titration.
[b] Compatibility in field grown plants: +, compatible; −, incompatible.
[c] Fiducial intervals calculated as in Finney (10).
[d] Confidence intervals calculated as in Steele and Torrie (25).
[e] Model represents data from two infectivity titrations.

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W. R. LANDIS ASSOCIATES, INC., Valdosta, GA