Bacterial Growth, Toxin Production, and Levels of Ornithine Carbamoyltransferase in Resistant and Susceptible Cultivars of Bean Inoculated with Pseudomonas phaseolicola

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ABSTRACT

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Time-course studies of growth and toxin production in culture were made with four isolates of *Pseudomonas phaseolicola:* G-50 and HB-36 (race 2) and HB-46 and HB-20 (race 1). In culture, all isolates increased approximately to the same population level (10^8-10^9 cells/ml) and all isolates except HB-20 produced toxin. Time-course experiments to measure growth, toxin production, and changes in the level of the enzyme, ornithine carbamoyltransferase (OCT), in bean leaf tissue, were made by inoculating two resistant and two susceptible bean cultivars with isolates HB-36 and HB-20 of the pathogen in separate experiments. In leaves of the susceptible bean cultivars "Resistant" Cherokee Wax and Red Kidney, both isolates increased to 10^9 cells per 1.2 cm^2 of leaf area from an initial level of 10^3 cells, but only HB-36 produced toxin. Also, in leaves of susceptible cultivars "Resistant" Cherokee Wax and Red Kidney, inoculated with HB-36, OCT activity averaged 53% and 71%, respectively, of that found in noninoculated leaves. In the resistant cultivars P.I. 150514 and GN #27, both isolates had much slower rates of multiplication than in susceptible cultivars; the highest number of bacterial cells observed was 10^5 cells per 1.2 cm^2 leaf area. In spite of this substantial increase in number of cells, no toxin was detected in resistant leaf tissues, including those that were inoculated with the toxin-producing isolate (HB-36) and there was no significant inhibition of OCT until 3 days after inoculation. Inhibition coincided with the appearance of the tissue browning and cell collapse characteristic of a hypersensitive reaction.

Additional key words: halo-blight, toxigenicity, phaseotoxin.

Pseudomonas phaseolicola (Burkh.) Dowson, the incitant of bean halo-blight, multiplies in inoculated bean (*Phaseolus vulgaris* L.) plants of both susceptible and resistant cultivars, although it multiplies less extensively in the latter (1, 4). In inoculated susceptible cultivars the proliferation of the pathogen results in localized chlorosis, localized water soaking, and systemic invasion of the host (4). However, in resistant cultivars these symptoms are not produced. It is known that chlorosis in an infected susceptible plant is caused by an extracellular toxin (phaseotoxin) produced by the pathogen (3, 8, 9). Therefore, the absence of at least the chlorosis part of the syndrome in inoculated resistant plants indicates that even though multiplication occurs, toxin is not produced. Recently, Patil et al. (7) isolated a toxinless ultraviolet-induced mutant of *P. phaseolicola* which multiplies as well in susceptible cultivars as does the toxin-producing wild type. It does not, however, produce chlorosis and does not attack the plant systemically. Because the only observable difference between the mutant and the wild type is in the ability of the wild type to produce toxin, it was proposed (7) that toxigenicity and the ability to invade systemically are causally related. Although we do not know how the two are related it is clear that phaseotoxin (6) is involved in more than induction of chlorosis in infected susceptible plants. In this study we investigate the time-course of toxin production by *P. phaseolicola* isolates in culture and in resistant and susceptible cultivars of bean. Further, we explored the hypothesis that in resistant tissues the production of phaseotoxin is suppressed and because of this no chlorosis or systemic symptoms are observed. The results show that toxin production in resistant plants indeed appears to be suppressed. A preliminary account of this work has been published previously (2).

MATERIALS AND METHODS

Bacterial isolates, culture, and inoculation techniques.—Patel and Walker (5) classified the isolates of *P. phaseolicola* into race 1 and race 2, on the basis of their pathogenicity to the bean cultivar Red Mexican UI-3. This cultivar was found resistant to all isolates of race 1 and susceptible to all isolates of race 2. In this study, for the purpose of examining the growth and toxin production by *P. phaseolicola,* two isolates of race 1 and two isolates of race 2 were selected. They are: isolates G-50 and HB-36 (race 2) [Supplied by J. Natti (deceased) and M. N. Schroth, respectively] and isolates HB-46 (Hagedorn race 1) and HB-20 (race 1). Isolates HB-36 and HB-20 were used in in vivo experiments on bacterial multiplication, toxin production, and changes in ornithine carbamoyltransferase (OCT) levels. Bean cultivars "Resistant" Cherokee Wax and Red Kidney

were the susceptible hosts and GN #27 and P.I. 150514 (supplied by D. Coyne, Department of Horticulture, University of Nebraska) were the resistant hosts. Routinely, 1.5 liters of Watanabe's (14) synthetic medium was inoculated in Fernbach flasks (2.8 liters) with an inoculum of 0.4×10^3 cells/ml taken from a 24-hour starter culture grown in the same medium. Similar starter cultures were also used to prepare inoculum (8.0×10^3 cells/ml) for spraying the plants.

Bean plants of uniform size were used 5-6 hours after their primary leaves unfolded, usually 5-6 days after planting the seeds. Cell suspensions were sprayed under pressure 70.5-149 g/cm^2 (1-2 psi) on the abaxial surface of the leaves until uniform water congestion was observed. Plants of the same age sprayed with sterile distilled water served as controls. The plants were incubated in a growth chamber with 16 hours of light (intensity 16,146 lux, fluorescent and incandescent) at 24 C and 8 hours of darkness at 22 C.

Estimations of bacterial populations. — Bacterial populations in cultures were determined by measuring the turbidity at 425 nm. Such determinations were made every 24 hours for 5 days. Simultaneously, viable cell populations were determined in triplicate by making dilution plates of nutrient-dextrose agar, which contained 1 ml of a 1.0 per cent solution of tetrazolium chloride (TZC) per liter of the medium, and the colonies were counted after 72 hours of incubation at 28 C. To measure the increase in the bacterial population in inoculated leaves, three leaf disks, 1.2 cm^2 in leaf area were removed every 24 hours for 5 days. The leaf disks were surface sterilized with 0.2% Alconox (2 minutes) followed by 70% EtOH (15 seconds), washed and ground in sterile distilled water and plated on TZC plates.

Toxin and enzyme assays.—The ability of bacterial isolates to produce phaseotoxin was determined as follows. Culture filtrates (100 ml) from cultures of each of the isolates were centrifuged (4,900 *g*, 10 minutes) and the supernatants concentrated to 5 ml in vacuo at 50 C. Nine volumes of methanol were added to each concentrate, the precipitate was removed by centrifugation, and the supernatant evaporated. The residue was dissolved in 2 ml of water and nine volumes of methanol were again added to it. After repeating the above procedure once more the final residue was dissolved in 1.5 ml of water and aliquots of this solution were bioassayed to determine toxin units. To estimate toxin content of leaf disks taken from inoculated plants, they were ground individually in 90% methanol, centrifuged (4,900 *g*, 10 minutes) and the supernatant evaporated to dryness at 50 C in vacuo. The residue was dissolved in 1 ml of water and 9 ml of methanol were added to it. After centrifugation and evaporation of the supernatant, residue was dissolved in 1 ml of water and the methanol extraction repeated once more. The final aqueous aliquots were tested for biological activity. The bioassay involved testing the inhibitory effect of solutions on the OCT activity. Patil et al. (8, 9) have previously reported that phaseotoxin is a potent inhibitor of OCT. The forward OCT assay described previously (13) was used in this study to calculate toxin units. One unit of toxin is that amount which produces a 50% inhibition of OCT under standard assay conditions (13).

To determine the levels of OCT in leaves of control plants and in those of inoculated resistant and susceptible plants, leaf disks were removed, extracted by grinding in tris-mercaptoethanol buffer (0.05M, pH 8.5) centrifuged, and the supernatant dialyzed overnight against the same buffer. Aliquots of this enzyme were used in the OCT assay and the level of OCT of various samples was determined as described (13). One unit of OCT has been described as that amount of enzyme which catalyzes the formation of 1 μmole of citrulline (from ornithine) in 10 minutes under standard assay conditions. The level (units) of extractable OCT in noninoculated leaves was determined first and assigned a value of 100%. The levels of OCT extracted from inoculated leaves were then subtracted from the above value and expressed (Table 1) as inhibition of OCT activity. These were transformed to the arcsin values and were subjected to standard analysis of variance. Means were separated by Duncan's multiple range test (12).

RESULTS

Cell growth and toxin production in culture.—All four isolates, G-50 and HB-36 (race 2) and HB-46 and HB-20 (race 1), showed roughly the same maximum growth (9×

TABLE 1. Changes in ornithine carbamoyltransferase (OCT) in resistant and susceptible bean leaves inoculated with toxin-producing (HB-36) and nontoxin-producing (HB-20) isolates of *Pseudomonas phaseolicola*

Bean cultivar	Bacterial isolate	Resistance (−) or susceptibility (+)	Inhibition (%) of OCT activity[z]				
			Day 1	Day 2	Day 3	Day 4	Day 5
P.I. 150514	HB-36	−	37 a	16 cd	26 c	31 d	40 c
	HB-20	−	14 d	10 d	10 e	26 e	36 c
GN #27	HB-36	−	39 a	19 c	18 d	21 f	15 e
	HB-20	−	24 bc	10 d	6 e	6 g	6 f
"Resistant" Cherokee Wax	HB-36	+	0 e	71 b	62 b	68 b	66 b
	HB-20	+	0 e	0 e	21 cd	40 c	66 b
Red Kidney	HB-36	+	28 b	84 a	82 a	81 a	78 a
	HB-20	+	22 c	22 c	20 cd	21 f	21 d

[z]Each value is a mean of three leaf disk replications. The levels (units) of OCT extracted from inoculated leaves were subtracted from that found in noninoculated leaves (100%) and expressed as inhibition of OCT activity. Means not followed by the same letter within a column differ significantly, $P = 0.05$, as determined by Duncan's multiple range test.

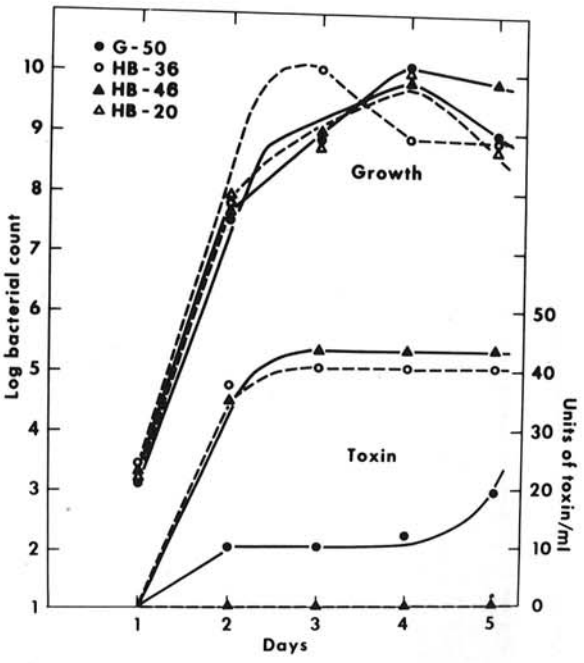

Fig. 1. Growth and toxin production by isolates G-50 and HB-36 (race 2) and HB-46 and HB-20 (race 1) of *Pseudomonas phaseolicola* in Watanabe's (14) medium.

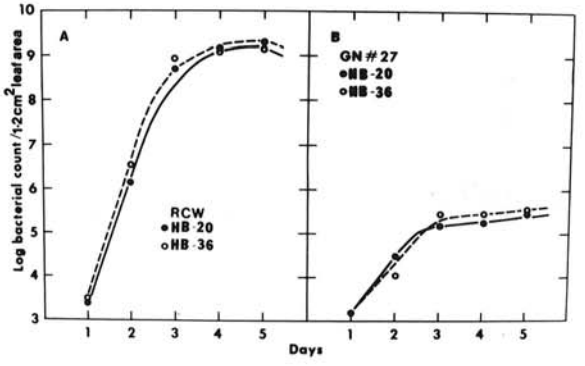

Fig. 2-(A, B). Growth of isolates HB-20 (race 1) and HB-36 (race 2) of *Pseudomonas phaseolicola* in **A**) "Resistant" Cherokee Wax (RCW) (susceptible), and **B**) GN #27 (resistant) cultivars of bean.

10^9 cells/ml) (Fig. 1). The isolates varied in their ability to produce phaseotoxin in culture (Fig. 1). Isolates HB-36 and HB-46 produced roughly two times more toxin than G-50; isolate HB-20 produced no toxin. Most of the toxin from HB-36 and HB-46 was produced during the first 24 hours, with minor increases between 48-72 hours. No increase in toxin was observed thereafter.

Growth, toxin production, and changes in levels of OCT in inoculated susceptible and resistant bean plants.—One isolate of race 1 (HB-20) and one isolate of race 2 (HB-36) were used in these studies. Their selection was based on the differences in ability to produce phaseotoxin in culture. In leaves of susceptible cultivars Red Kidney and "Resistant" Cherokee Wax both isolates increased at about the same rate from an initial population of 10^3 cells to about 10^9 cells per 1.2 cm^2 leaf area (Fig. 2-A and 3-A). The isolates varied in the kind of symptoms they induced: HB-20 produced water-soaked lesions and localized chlorotic halos in the inoculated primary leaves, whereas HB-36 caused systemic chlorosis accompanied by stunting of the entire plant. Only isolate HB-36 produced toxin in the susceptible cultivars (Table 2). Toxin was first detected on the second day after inoculation in Red Kidney and on the third day in "Resistant" Cherokee Wax. More toxin was produced in the leaves of "Resistant" Cherokee Wax than in Red Kidney. Levels of OCT examined on time sequence (Table 1) showed that 71% of the enzyme in HB-36 inoculated Red Kidney leaves and 84% of the enzyme in HB-36 inoculated "Resistant" Cherokee Wax leaves was inhibited on the second day after inoculation, while only 16 and 19% of OCT was inhibited in the leaves of resistant GN #27 and P.I. 150514, respectively. On the other hand, on the second day after inoculation in HB-20 inoculated susceptible and resistant leaves, maximum inhibition of OCT was only 22%. Only in the susceptible leaves inoculated with HB-36, was consistently higher inhibition of the enzyme observed than in resistant leaves until the fifth day after inoculation.

In resistant leaves, both HB-20 and HB-36 multiplied at a much slower rate than in the susceptible leaves and reached a maximum population of 10^5-10^6 cells per leaf disk (Fig. 2-B and 3-B). In contrast to the symptoms seen in the susceptible cultivars, no water soaking, chlorosis, or stunting was observed in resistant cultivars. Instead, between 48-72 hours after inoculation general browning and cell collapse on the lower surface were observed. Toxin was not detected at any time in resistant leaves inoculated with either isolate (Table 2). The level of OCT decreased very little until the third day after inoculation, when greater decrease in OCT in these resistant leaves occurred than in the previous days (Table 1). This decrease was correlated with the appearance of the hypersensitive response by the host.

DISCUSSION

The time sequence studies on the multiplication of *P. phaseolicola* in culture and in susceptible plants show that the temporal pattern and extent of growth in either medium is essentially the same (Fig. 1, 2-A, 3-A). It is noteworthy that the isolate, HB-20 (race 1) multiplies roughly to the same extent as HB-36 (race 2) in susceptible cultivars even though unlike the latter it fails to become systemic and is thus less virulent. Omer and Wood (4) have previously reported similar results on growth of *P. phaseolicola* isolates. Schroth et al. (11) reported that several naturally occurring isolates of race 1 produced the same number of local lesions as did race 2 isolates, but they did not become systemic. These findings on pathogenic multiplication in vivo indicate that regardless of race, the rate of multiplication of all isolates of *P. phaseolicola* in a given cultivar, is about the same.

When the results of experiments on the ability of different *P. phaseolicola* isolates to produce toxin in culture (Fig. 1) and in susceptible plants (Table 2, supported by the OCT inhibition data in Table 1) are

TABLE 2. Toxin production by isolate HB-36 (race 2) of *Pseudomonas phaseolicola* in resistant (P.I. 150514 and GN #27) and susceptible (Red Kidney and "Resistant" Cherokee Wax) cultivars of bean

Bean cultivar	Bacterial isolate	Resistance (−) or susceptibility (+)	Units of toxin[a] produced in one leaf disk (1.2 cm^2) leaf area				
			Day 1	Day 2	Day 3	Day 4	Day 5
P.I. 150514	HB-36	−	0	0	0	0	0
	HB-20	−	0	0	0	0	0
GN #27	HB-36	−	0	0	0	0	0
	HB-20	−	0	0	0	0	0
Red Kidney ± Standard deviation[b]	HB-36	+	0	550[b] ±69.5	652[b] ±33.8	652[b] ±25.4	680.25[b] ±51.4
	HB-20	+	0	0	0	0	0
"Resistant" Cherokee Wax ± Standard deviation[b]	HB-36	+	0	0	832.5[b] ±11.7	1312.5[b] ±28.7	1479[b] ±67.2
	HB-20	+	0	0	0	0	0

[a]One unit of toxin is that amount of toxin which gives 50% inhibition of ornithine carbamoyltransferase (OCT) under standard assay conditions (13).

[b]Each value is the mean ± standard deviation of toxin concentration of three leaf disk replications.

contrasted to their ability to produce systemic symptoms, a strong correlation between virulence and toxigenicity emerges. HB-36 and G-50, both race 2 isolates, produced large quantities of toxin both in culture and in susceptible leaf tissue [data for in vivo toxin production by G-50 (Gnanamanickam and Patil, *unpublished*)] and also became systemic. In contrast, HB-20 (race 1) produced no toxin either in culture or in susceptible tissues and failed to become systemic. Unlike HB-20, however, HB-46 which has also been designated as race 1, did elaborate the toxin and also produced systemic symptoms in susceptible cultivars "Resistant" Cherokee Wax and Red Kidney. Thus, there appears to be a contradiction between the behaviour of the two race 1 isolates used in this study.

The contradiction between the behaviour of these isolates, however, is not surprising because the original classification of the two races was based on the use of only one differential bean cultivar, Red Mexican UI-3 (5). When Schroth et al. (11) studied the pathogenicity of the isolates of races 1 and 2 of *P. phaseolicola* on several bean cultivars, they observed that the race 1 isolates were generally less virulent than race 2 isolates. However, there were many exceptions in both races and they concluded that neither the race 1 nor the race 2 group isolates were homogeneous in virulence. In the study of Schroth et al. (11) HB-46 behaved like a race 2 isolate and produced susceptible reaction in several cultivars. In the current study also HB-46 inoculated plants of "Resistant" Cherokee Wax and Red Kidney showed severe systemic chlorosis and the pathogen produced abundant toxin in culture. Thus, HB-46 is similar to G-50 and HB-36 in its behaviour, and thus appears to be misplaced as a race 1 isolate.

Although the maximum growth of *P. phaseolicola* isolates in resistant cultivars was much lower than in susceptible cultivars there was a net gain of at least two orders of magnitude in cell numbers in the former. A comparison of toxin content of susceptible cultivar Red Kidney inoculated with HB-36 which contained roughly the same number of cells (second day after inoculation) as the inoculated P.I. 150514 (on the third day after inoculation), showed that the former contained 550 units of toxin per unit leaf area, whereas none was detected in the latter. This indicates that toxin was not produced in resistant tissues. The fact that no significant OCT inhibition (8) was observed in these tissues (Table 1) lends further support to this contention. It is possible that the toxin was produced but was degraded in the leaf tissues. This appears unlikely, however, because isolated toxin induces chlorosis in both the susceptible as well as resistant leaf tissues (10) indicating that the latter do not degrade the toxin.

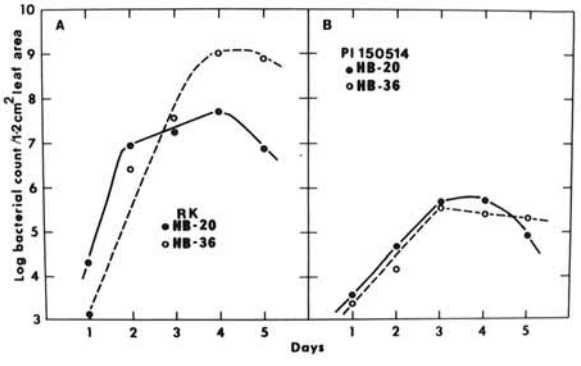

Fig. 3-(A, B). Growth of isolates HB-20 (race 1) and HB-36 (race 2) in **A)** Red Kidney (RK) (susceptible) and **B)** P.I. 150514 (resistant) cultivars of bean.

The finding that the resistant tissues suppress toxin production by *P. phaseolicola* when taken together with the inability of the pathogen to become systemic in such tissues corroborates the previous report that toxigenicity is essential for the ability to invade the plant systemically (7), thus implicating toxin in the pathogenicity of *P. phaseolicola* for its host.

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