Isoflavonoid Phytoalexins and Nonhypersensitive Resistance of Beans to *Xanthomonas campestris* pv. *phaseoli*

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ABSTRACT

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Bean phytoalexins, phaseollinisoflavan and kievitone, strongly inhibited *Xanthomonas campestris* pv. *phaseoli* in liquid culture while phaseollin and coumestrol were not inhibitory. When phytoalexin accumulation and xanthomonad multiplication in unifoliate leaves of the susceptible bean cultivar Dark Red Kidney and the resistant bean cultivar Tara were compared, none of the known isoflavonoid phytoalexins were found in Dark Red Kidney tissue exhibiting typical common blight symptoms. Hypersensitively reacting tissue of Tara contained significant amounts of phaseollin and phaseollinisoflavan, lower amounts of coumestrol, and an unidentified, moderately inhibitory coumestan. However, Tara tissue which did not react hypersensitively did not contain detectable levels of any of the isoflavonoids 5 days after inoculation with 10^6 colony-forming units of *X. campestris* pv. *phaseoli* per milliliter. At this time populations of *X. campestris* pv. *phaseoli* had attained stable stationary levels, indicating that resistance had been induced and was well established. The limited antibacterial activity of phaseollin and coumestrol and the absence of all four characterized isoflavonoids in nonhypersensitive resistant Tara tissue suggest that the four phytoalexins studied do not contribute significantly to nonhypersensitive resistance of beans to *X. campestris* pv. *phaseoli*.

Isoflavonoid phytoalexins are thought to contribute to the resistance of legumes to several fungal pathogens because such compounds are antimicrobial in vitro and they accumulate in infected plant tissues (30). However, comparatively few studies on the role of phytoalexins in bacterial diseases have been conducted, and the results of such investigations have varied considerably. Therefore, it may be premature to conclude that phytoalexins are involved in general disease resistance.

Some isoflavonoid phytoalexins have been reported to inhibit bacterial phytopathogens (15,23,33), but most studies have shown that the majority of the phytoalexins tested are not significantly inhibitory to most of the bacterial phytopathogens examined (6,8,14,16,24,28,32,33). The relative tolerances of the various pathogens to a given phytoalexin have not been correlated with differences in compatibility of the pathogens with the plant producing that phytoalexin. Moreover, investigators disagree about the activity of particular isoflavonoids (eg, phaseollin, kievitone, or coumestrol) against the same bacterial pathogens (14,15,23,33). Differences in bioassay methods may in part account for the discrepancies.

Phaseollin, glyceollin, and several other isoflavonoids have been found in necrotic hypersensitive reactions (HR) induced by bacteria in beans and soybeans (15,22–24). However, Webster and Sequeira (32) did not detect the characterized phytoalexins in resistant bean hypocotyls inoculated with low (10^4 colony-forming units [CFU] per milliliter) concentrations of *Pseudomonas syringae* that did not induce HR. The relationship between the severe histological and physiological alterations in the host during HR and the changes in host processes during interactions with lower numbers of bacteria is not known. Host constituents released by tissue injury can induce phytoalexin synthesis, and necrotic tissue can act as a sink for isoflavonoids synthesized in adjacent healthy tissue (17,18). Therefore, extrapolation from data that show phytoalexin accumulation in the severe necrosis associated with HR to infected tissues resistant in the absence of HR may be misleading. Thus, phytoalexin accumulation in plants inoculated with low numbers of bacteria needs to be examined before the role of phytoalexins in nonhypersensitive resistance can be evaluated.

We previously found that, unlike most of the phytopathogenic bacteria examined, *Xanthomonas campestris* pv. *phaseoli* was strongly inhibited by phaseollinisoflavan and kievitone, two of its host's phytoalexins (33). Because of this pathogen's high sensitivity to these two compounds and because of the controversy regarding the antibacterial activity of two additional bean phytoalexins, phaseollin and coumestrol, we examined the accumulation of all four isoflavonoids relative to the multiplication of *X. campestris* pv. *phaseoli*.

The objective of this study was to determine whether increases in these characterized phytoalexins were sufficient to account for the resistance of certain bean cultivars to this pathogen when HR was not induced. A preliminary report of these results has been published (34).

MATERIALS AND METHODS

Media. Potato-dextrose agar (PDA), nutrient broth (NB), nutrient agar (NA), and nutrient agar plus 1% glucose and 0.5% yeast extract (NAGY) were used as growth media. All culture media were obtained from Difco Laboratories (Detroit, MI 48232). For some experiments, culture media were supplemented after autoclaving with filter-sterilized streptomycin sulfate (Str) (Sigma Chemical Co., St. Louis, MO 63178) to a final concentration of 250 μg/ml. Streptomycin-amended NAGY medium is hereafter designated Str-agar.

Bacteria. *Xanthomonas campestris* pv. *phaseoli* G32, a highly virulent isolate used in a previous study (3), served as the reference strain and as the parent for obtaining Str-resistant mutants. Spontaneous Str-resistant mutants were obtained at frequencies of 2.4×10^{-8} and 5.8×10^{-9} in separate trials by plating a water suspension of a 16-hr NA culture of G32 on Str-agar and incubating at 25 C for 96 hr. Fourteen mutants were purified by repeated single-colony isolation on Str-agar and then tested for virulence by injecting primary leaves of 14-day-old *Phaseolus vulgaris* L. 'Dark Red Kidney.' Three concentrations (10^4, 10^6, and 10^8 CFU per milliliter) per strain were tested, and symptom development was compared daily with that occurring in beans similarly inoculated with strain G32. Under the controlled environmental conditions routinely employed, strains SR36 (the most aggressive mutant obtained) and G32 were both highly virulent. The rate and severity of symptom development were identical for both strains at all inoculum concentrations.

G32 and SR36 were maintained on PDA and PDA plus 250 μg of Str per milliliter, respectively, at 6 C. G32 was subcultured at 3-mo

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intervals, but SR36 required monthly transfer. Periodic streaking on NAGY or Str-agar to check colony morphology revealed small, nonmucoid, weakly virulent colonies in SR36 after repeated subculturing (9–12 mo), but no such variants were observed in G32 throughout the study. Only wild-type, large, mucoid colonies were used as inoculum in experiments.

Plants. Seeds of *Phaseolus vulgaris* L. 'Dark Red Kidney' (Agway, Inc., Syracuse, NY 13201) and cultivar Tara (a generous gift from D. P. Coyne, Dept. of Horticulture and Forestry, University of Nebraska, Lincoln 68583) were surface sterilized in 0.5% sodium hypochlorite for 3 min, rinsed well with tap H_2O, and incubated in rolled moist paper towels for 2 days. Individual germinated seeds were planted in sterile potting mix (loam:peat:sand [1:1:1, v/v]) in 8-cm-diameter clay pots. Plants were grown in a controlled environment chamber maintained at 27 C, 14.5-hr photoperiod, 15,000 lux at plant height, and ambient relative humidity (about 70%). Seedlings $\geq$12 days old were fertilized daily with Hoagland's nutrient solution (19).

Bacterial multiplication in vivo. Inocula containing 2.8×10^4, 2.8×10^6, or 2.8×10^8 CFU per milliliter were prepared by suspending SR36 cells from 16-hr Str-agar cultures in H_2O and diluting appropriately after determining the optical densities at 580 nm. Fully expanded primary leaves of 11-day-old Dark Red Kidney or Tara beans were injected with inocula by using syringes fitted with 0.46-mm-diameter (26-gauge) needles until approximately 75% of the leaf area was confluently water-soaked. Inoculated zones were outlined with indelible ink, and plants were returned to the growth chamber. Water-soaking disappeared within 30 min and no visible symptoms developed in leaves injected with H_2O only.

At 5 hr after inoculation, and subsequently at daily intervals, twenty 9-mm-diameter disks were excised from each plant and homogenized in 3.0 ml of 50 mM sodium phosphate buffer, pH 7. A serial decimal dilution series was made in buffer, and bacteria were plated on Str-agar. Colony counts were made after 96 hr of incubation at 25 C, and data were expressed as $\log_{10}$ CFU per square centimeter of leaf. Two replicate plants of each cultivar were used at each sampling. Inoculum concentrations of 2.8×10^8 CFU per milliliter were tested in three experiments, 2.8×10^6 CFU per milliliter in four experiments, and 2.8×10^4 CFU per milliliter in one experiment.

The multiplication of strains G32 and SR36 in each cultivar was compared by similar methods in one experiment per cultivar. Inoculum concentrations were 4.5×10^6 and 2.8×10^6 CFU per milliliter for G32 and SR36, respectively.

Growth curves from single experiments were analyzed statistically by comparing mean populations in the two treatments (eg, cultivar or strain) by *t*-test when water-soaked tissue and necrosis were first observed in Dark Red Kidney and using pure error as a measure of the variance. Due to the absence of symptoms in Tara beans inoculated with $\leq 10^6$ CFU per milliliter, populations of G32 and SR36 in Tara were compared by *t*-test at 6 and 11 days, the respective approximate midpoint and final point of the recorded stationary phase. Data from experiments in which 10^6 or 10^8 CFU per milliliter were used to compare SR36 multiplication in the two cultivars also were analyzed by pooling all replicates per sampling time per cultivar from all experiments with the same inoculum concentration. Means of pooled replicates were plotted over time to generate composite growth curves for each cultivar, and 95% confidence intervals were calculated. Mean SR36 populations in the two cultivars were compared by *t*-test when water-soaking and necrosis were first observed in Dark Red Kidney.

Isoflavonoids. Phaseollinisoflavan was extracted from diseased cultivar Topcrop beans by methods described previously (31) and purified by thin-layer chromatography (tlc) in the following solvent systems: (A) benzene:methanol (MeOH) (9:1, v/v), unsaturated; (B) chloroform:MeOH (25:1, v/v), unsaturated; (C) benzene:ethyl acetate:MeOH (25:8:4, v/v), unsaturated, or toluene:ethyl formate:formic acid (7:2:1, v/v), unsaturated; and (D) hexane:acetone (3:1, v/v), unsaturated. Phaseollin, kievitone, and coumestrol were obtained and stored as described previously (33). The identity of coumestrol was confirmed by mass spectral analysis (H. D. VanEtten and J. G. Wyman, *unpublished*). Isoflavonoid concentrations were determined from their ultraviolet absorbances in ethanol and from published extinction coefficients (2,7,9,26).

Phytoalexin accumulation in infected tissue. Three separate areas (3.5 cm in diameter) per unifoliate leaf were inoculated by the same methods employed for growth-curve studies. To confirm that bacterial multiplication followed patterns predicted by the growth-curve studies, xanthomonad populations in similarly inoculated plants were determined every 48 hr and at the time the tissue was harvested. At various time intervals, diseased tissue was excised, lyophilized, and either extracted immediately or stored at −20 C.

Lyophilized tissue (1.0 g dry weight) was vacuum-infiltrated with 50 ml of redistilled ethanol (EtOH), and the ethanolic solution was filtered through Whatman GF/A glass-fiber paper (Whatman, Inc., Clifton, NJ 07014). The tissue was comminuted in 100 ml of redistilled EtOH for 1 min, filtered, and re-extracted two or three times. The combined EtOH eluates were mixed with 100 ml of H_2O and reduced in vacuo at 38 C to the aqueous phase (about 80–90 ml), which was partitioned three or four times with approximately 250 ml of chloroform. The combined $CHCl_3$ fractions were dried in vacuo. The residue was transferred to a small vial with several washes of EtOH and $CHCl_3$, dried under a stream of air, and stored at −20 C. An uninoculated tissue control (1.0 g dry wt) and a recovery efficiency control (1.0 g dry wt of uninoculated tissue plus 300 μg each of phaseollin, phaseollinisoflavan, coumestrol, and kievitone) were coextracted with each inoculated tissue sample.

Residues were dissolved in 300–400 μl of a 3:1 EtOH:$CHCl_3$ mixture. Volumes containing 0.25–0.5 g dry wt equivalents were spotted on 0.25 mm Whatman LK5F silica gel plates (Whatman, Inc.) that had been prerun twice in MeOH in each of two dimensions. Plates were developed in ethyl ether:hexane (5:1, v/v), unsaturated (solvent system E), rotated 90°, and redeveloped in solvent system A. Phytoalexin standards were included in each dimension. The silica gel from all quenching and fluorescent areas on the tlc plate that were visible under near- or far-ultraviolet (UV) light were eluted with 1.0 ml of redistilled EtOH. Areas corresponding to R_fs of each of the known phytoalexins were eluted similarly even if no spots were detected in these zones. The eluates were filtered through glass wool, and their UV absorbance spectra were determined with a Perkin-Elmer, Hitachi 200 UV spectrophotometer. Reported concentrations are corrected for efficiencies of extraction. Plates from which UV-detectable spots had been removed were sprayed with diazotized *p*-nitroaniline reagent to detect additional phenols (27).

Bioassays. The in vitro sensitivity of G32 and SR36 to four isoflavonoid phytoalexins in liquid medium was determined by previously described methods (33) modified as follows. Inocula were grown on NAGY and Str-agar for G32 and SR36, respectively, for 13–17 hr at 25 C. NB was substituted for modified Starr's medium. Isoflavonoids were dissolved in dimethylsulfoxide (DMSO) and added to the media to give a final DMSO concentration of 1.8%. The treated cultures (two or three replicates per treatment) were incubated with reciprocal shaking (210 strokes per minute) for 21 hr. The optical densities of diluted samples were measured with a Perkin-Elmer, Hitachi 200 UV spectrophotometer against a blank of 0.22 ml of NB plus 1.0 ml of H_2O. Phaseollin, phaseollinisoflavan, and kievitone were tested in three experiments and coumestrol in two experiments.

For the tlc bioassay, tlc plates spotted with 0.15 g dry wt equivalents of diseased tissue extracts were developed in two dimensions as above, scanned under UV light, and dried for 10–15 min. SR36 cells from Str-agar cultures grown for 14–16 hr at 25 C were suspended in H_2O, and 1.0 ml of the resultant turbid suspension was mixed rapidly with 9 ml of molten (52 C) tlc medium (NB plus 2% glucose, 0.5% yeast extract, and 0.7% agar). Dried tlc plates were sprayed with the mixture until a uniform, thin, wet film developed, incubated at 25 C in glass trays lined with moist paper towels, and misted daily with the same medium minus agar to enhance growth. On wet plates areas of inhibition appeared whiter and more depressed than areas where growth was not inhibited. On plates dried after 96 hr of incubation, zones of strong inhibition appeared white, whereas other areas were a uniform yellow

resulting from growth of the yellow-pigmented xanthomonad. Areas of weak inhibition were a light yellow that were readily distinguished from the darker yellow in areas where there was no inhibition.

To determine the minimum amounts of the known phytoalexins that would cause detectable zones of inhibition, several quantities (0.25–14 μg) of each of the phytoalexins were applied in 4- to 6-mm-diameter spots on a prerun silica gel plate that was then sprayed with the bacteria-seeded medium and incubated as above. Only readily detectable, completely white zones were scored as positive.

RESULTS

Mutant characterization. Preliminary studies indicated that the plating efficiency of the *Xanthomonas campestris* pv. *phaseoli* mutant SR36 was unaffected by the concentration of streptomycin used in the antibiotic-amended medium. After 2 wk of repeated subculturing every 48 hr in NB or 2 wk after inoculating Dark Red Kidney and Tara beans with suspensions of 10^4 CFU per milliliter, the plating efficiencies of SR36 from all sources on NAGY versus Str-agar were identical, indicating that the Str marker was stable under nonselective conditions. Initial comparisons of SR36 and its parent, G32, showed that the two strains incite equally rapid and severe symptoms typical of common blight in susceptible Dark Red Kidney beans, whereas neither strain at inoculum concentrations of $\leqslant 10^6$ CFU per milliliter incited visible symptoms in Tara beans. The strains had equivalent doubling times in vitro (J. G. Wyman, *unpublished*) and their rates of logarithmic increase in planta were similar (Fig. 1). Although G32 and SR36 were equally virulent on the basis of disease severity and multiplied at similar rates, G32 multiplied to higher levels in planta in the one comparative experiment run (Fig. 1). The observed differences in stationary phase levels of the two strains in this experiment, however, were not statistically significant ($P = 0.05$) in either cultivar and may be somewhat exaggerated because counts of G32 on nonselective media may have included colonies of the numerous yellow-pigmented saprophytic bacteria present on bean leaves. In addition, in other experiments with inocula containing 10^6 CFU per milliliter, SR36 populations usually attained higher levels (Fig. 2B).

The sensitivities of G32 and SR36 to four isoflavonoids in vitro were similar (Table 1). Phaseollinisoflavan and kievitone significantly inhibited both strains, although the ED_{50} values for each compound were somewhat higher for G32 than for SR36. The other two compounds did not inhibit either strain significantly. The bioassay modifications resulted in more bacterial growth than was observed previously. However, the activities of the various isoflavonoids against G32 were similar to previously published results (33 and Table 1), although the ED_{50} values of phaseollinisoflavan and kievitone were higher and the relative toxicity of these two compounds was reversed. For three of the compounds the percentages of inhibition at the maximum concentrations tested tended to be lower than in the previous assay (33).

Bacterial multiplication in vivo. Multiplication of SR36 in primary leaves of both cultivars followed a typical sigmoid curve (Fig. 2). Regardless of the inoculum concentration used, the xanthomonad rapidly multiplied in Dark Red Kidney to $\geqslant 10^8$ CFU per square centimeter of leaf, at which time water-soaking was first observed. The period between the onset of water-soaking and subsequent necrosis varied inversely with the initial inoculum concentration. Subsequent symptoms included severe blighting of adjacent trifoliolate leaves and development of stem lesions. In Tara, however, periods of logarithmic increase were shorter, and maximum population levels attained strongly depended on inoculum concentration. Stationary populations in Tara were significantly lower than those in Dark Red Kidney for all inoculum concentrations ($P = 0.001$ for experiments employing 10^6 and 10^8 CFU per milliliter and $P = 0.05$ for the experiment using 10^4 CFU per milliliter). Unifoliate leaves of Tara injected with inocula containing 10^4 or 10^6 CFU per milliliter remained symptomless throughout the observation periods, and stationary phase populations of SR36 remained relatively constant. Within 20 hr after inoculation with 10^8 CFU of SR36 or the incompatible species *X. campestris* pv. *vesicatoria* or *Pseudomonas syringae* pv. *lachrymans* per milliliter, Tara leaves developed diffuse, gray-brown, necrotic hypersensitive lesions that became tan, papery, and confluent in most of the inoculated zone during the following 24 hr. The continued slow increase of SR36 after 20 hr may reflect in part the nonuniform distribution of the hypersensitive reactions within the inoculated zone. For example, in one of the three experiments employing 10^8 CFU per milliliter the tissue became necrotic earlier and a greater proportion of the inoculated zone became necrotic than in other trials. The SR36 populations increased less after HR and remained constant between 3 and 7 days after inoculation in this experiment (Fig. 2A).

Phytoalexin accumulation. The two-dimensional tlc system readily separated the four isoflavonoids from one another and from most of the contaminating plant pigments. In the tlc bioassay, zones of strong inhibition coincident with residual plant pigments were easily discerned on wet plates and frequently were surrounded by a ring of enhanced growth that aided their detection on dried plates. In the tlc bioassay, amounts as low as 0.25 μg of phaseollinisoflavan or kievitone and 0.75 μg of phaseollin completely inhibited the xanthomonad, whereas 5–10 μg of coumestrol were not inhibitory.

Untreated controls and H_2O-injected controls of either cultivar did not contain detectable levels of the characterized isoflavonoids. Hypersensitive necrotic tissue of Tara extracted 2 days after inoculation contained significant amounts of phaseollin and phaseollinisoflavan (Table 2). Coumestrol was not detected, but a substance with a UV spectrum typical of the coumestans was found at a concentration of 1.3 absorbance units at 343 nm (λ_{max}) per gram dry weight. This probable coumestan was not identified, but

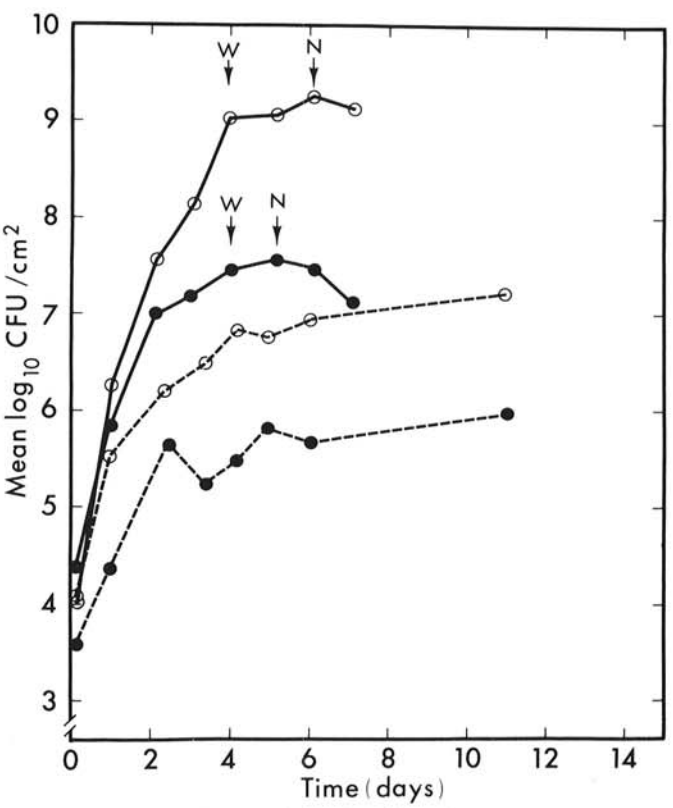
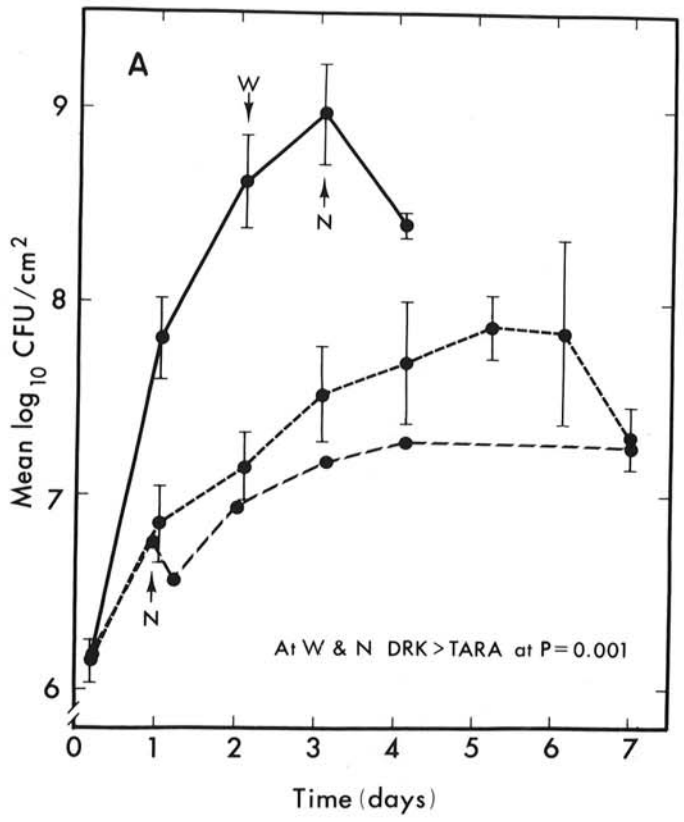
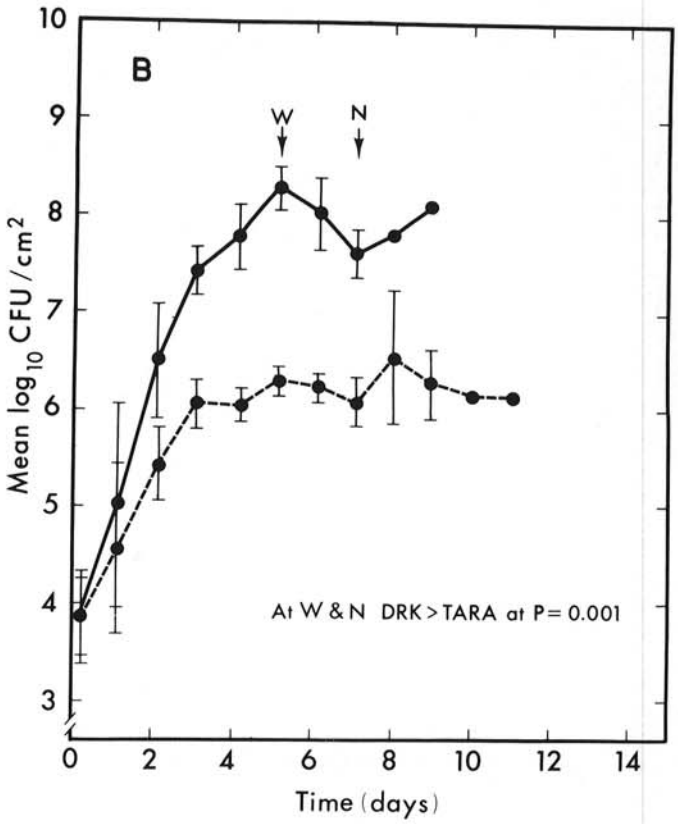


Fig. 1. Comparison of the multiplication of *Xanthomonas campestris* pv. *phaseoli* strains G32 (O) and SR36 (●) in primary leaves of bean cultivars Dark Red Kidney (——) and Tara (— —) beans inoculated with approximately 10^6 CFU per milliliter. Water-soaking (H) and necrosis (N) developed at the time indicated in Dark Red Kidney. Data were analyzed by *t*-test, using pure errors ($s_{overall}$) of 0.12 and 0.25 in experiments on Tara and Dark Red Kidney, respectively.





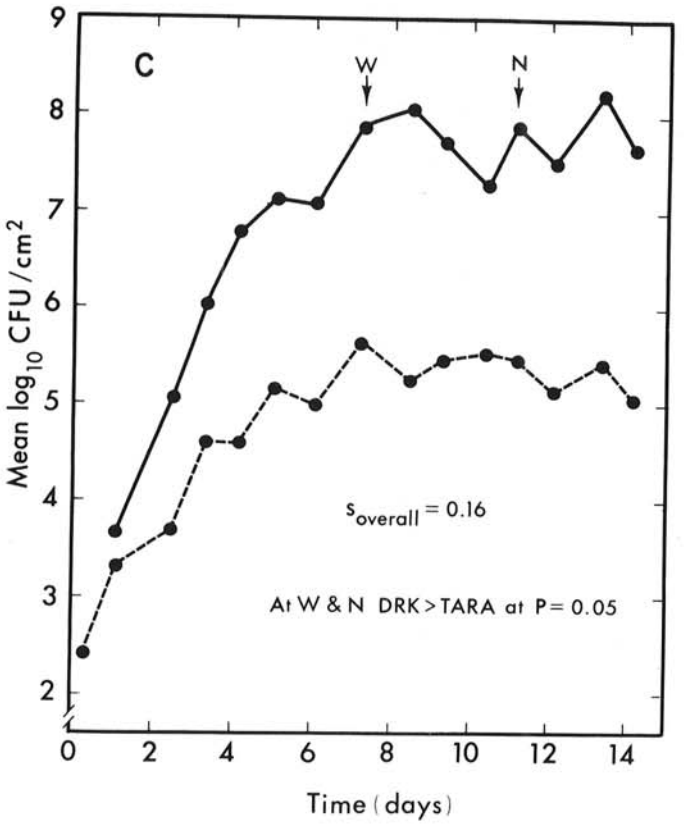


Fig. 2. Comparison of the multiplication of SR36 in unifoliolate leaves of bean cultivars of Dark Red Kidney (——) and Tara (— —). Data were analyzed by *t*-test when water-soaking (W) and necrosis (N) were first observed in leaves of Dark Red Kidney. Inoculum concentrations (CFU per milliliter) were **A**, 2.8×10^8; **B**, 2.8×10^6, and **C**, 2.8×10^4. Fig. 2A and B are composite growth curves; Fig. 2C is from one experiment. In Fig. 2A results of one experiment (— — —) are compared to the composite curve (——) for all experiments with inocula at 10^8 CFU per milliliter.

differed from coumestrol in several respects: tlc R_f values in solvent systems A and E (0.23, 0.54 for the unknown versus 0.36, 0.12, respectively, for coumestrol); UV absorbance spectrum (no 305 nm peak, $\lambda_{min} = 304$ nm for the unknown versus a 305-nm peak, $\lambda_{min} = 284$ nm for coumestrol); retention time in gas-liquid chromatographic analysis (T. Denny and J. G. Wyman, *unpublished*); and weak-to-moderate antibacterial activity by the unknown in the tlc bioassay versus no inhibition by coumestrol. A substance resembling kievitone in R_f and spectrum could not be positively identified due to impurities and its presence in low concentration. At 4 days, phaseollin and phaseollinisoflavan concentrations remained fairly high; coumestrol was present, but the unidentified coumestan and kievitone were not detected. Several other strongly inhibitory phenolic materials with R_f values of $\leqslant 0.5$ in each solvent system were found in extracts of tissue excised 2 and 4 days after inoculation. All zones of inhibition were associated with UV-detectable substances.

In contrast to HR tissue, nonhypersensitively reacting Tara leaves extracted 5 days after inoculation with 10^6 CFU per milliliter did not contain detectable levels of any of the four characterized phytoalexins in any of three experiments. Even though SR36 had reached stable stationary levels by 5 days, none of the numerous UV-detectable substances present in extracts at that time were inhibitory in the tlc bioassay or had UV spectra similar to those of known phytoalexins. Tlc bioassays of extracts of similar nonhypersensitively reacting resistant Tara tissue at 8 days revealed weakly and strongly inhibitory materials with mobilities similar to those of phaseollin and phaseollinisoflavan, respectively. These substances developed the characteristic color reactions associated with those isoflavonoids when sprayed with diazotized *p*-nitroaniline. However, their UV absorbance spectra were not characteristic of the two phytoalexins, possibly due to the presence of impurities and the low amounts present.

Similarly inoculated, susceptible Dark Red Kidney tissue developed typical common blight symptoms and was collected at the same times that Tara leaves were sampled. In seven different experiments, Dark Red Kidney tissues did not contain detectable levels of any of the characterized phytoalexins or other inhibitory materials.

DISCUSSION

The absence of symptoms in Tara leaves inoculated with 10^6 *X. campestris* pv. *phaseoli* cells per milliliter and the development of hypersensitive reactions in response to higher numbers of xanthomonads are consistent with Coyne and Schuster's classification of the cultivar Tara as "common blight tolerant" (synonymous with resistant [D. P. Coyne, *personal communication*]) (1,3,4). Other investigators who rated cultivar Tara as "moderately resistant" may have underestimated its resistance because they either used high inoculum concentrations that could have induced hypersensitive reactions and rating scales that relied solely on the degree of necrosis on inoculated leaves 3 wk after inoculation (10) or they assessed disease at the pod stage when cultivars deriving their resistance from G. N. Nebraska #1 selection 27 (the source of Tara's resistance) are more susceptible (5,35). The significantly lower stationary phase levels of the xanthomonad obtained in Tara in response to all inoculum concentrations and the dependence of stationary phase levels on initial inoculum concentrations agree with patterns of multiplication of cells of incompatible *Pseudomonas* and *Xanthomonas* species in beans and cotton (11,12,21,25,29). These results thus confirm that Tara is resistant to *X. campestris* pv. *phaseoli*. The resistance appears to depend more on induced factors than on preformed, constitutive factors because the observed resistance was expressed only after a period of logarithmic increase at rates only somewhat lower than those observed in susceptible Dark Red Kidney.

The response of SR36, G32 (Table 1), and two additional virulent wild-type strains (J. G. Wyman, *unpublished*) to the phytoalexins in liquid growth media confirm previous findings that *X. campestris* pv. *phaseoli* is highly sensitive to the bean phytoalexins phaseollinisoflavan and kievitone, but tolerant of phaseollin and coumestrol, under the in vitro conditions that were employed (33). However, all the isoflavonoids except coumestrol gave zones of inhibition in the tlc bioassay. It was not determined whether the active substances in the tlc bioassay were the phytoalexins themselves or breakdown products produced as a result of prolonged contact with silica gel. Regardless of the basis for the growth inhibition of *X. campestris* pv. *phaseoli*, the tlc bioassay proved to be a highly sensitive means of monitoring the accumulation of three of the four isoflavonoid phytoalexins studied.

None of the four isoflavonoid phytoalexins studied was present in necrotic Dark Red Kidney leaf tissue at levels detectable by either tlc bioassay or spectrophotometric analysis. This is consistent with published findings of their absence or extremely low concentration in various susceptible interactions of beans with other compatible bacterial species (22–24,28). Hypersensitively reacting tissue of Tara, however, contained significant amounts of phaseollin and phaseollinisoflavan and lower amounts of coumestrol, demonstrating that Tara is capable of synthesizing isoflavonoid phytoalexins in response to high humbers (10^8 CFU per milliliter) of *X. campestris* pv. *phaseoli*. As in other bacterially induced hypersensitive reactions of bean, necrotic Tara tissue apparently contained an unidentified coumestan in addition to coumestrol (24). The moderate antibacterial activity of this related compound may account in part for the controversy regarding the toxicity of coumestrol to microorganisms (15,24,33).

Holliday et al (20) recently reported circumstantial evidence that glyceollin accumulates in soybean tissue responding in a nonhypersensitive resistant manner to *Pseudomonas syringae* pv.

TABLE 1. Effect of four isoflavonoids from bean on growth of *Xanthomonas campestris* pv. *phaseoli* in liquid media

		Strain G32				Strain SR36	
		Modified Starr's medium[a]		Nutrient broth[b]		Nutrient broth[b]	
Isoflavonoid	Maximum conc. tested (μM)	ED_{50} (μM)	Inhibition at maximum conc. tested (%)	ED_{50} (μM)	Inhibition at maximum conc. tested (%)	ED_{50} (μM)	Inhibition at maximum conc. tested (%)
Phaseollin	140	>140	22	>140	9	>140	−14[c]
Phaseollin-isoflavan	300	40	100	82	100	64	100
Coumestrol	140	>140	17	>140	5	>140	−2
Kievitone	300	22	99	116	94	84	96

[a] Bacteria were grown in 0.22 ml of isoflavonoid-amended modified Starr's medium (33) at 25 C at 185 strokes per minute on a reciprocal shaker for 26 hr. Samples then were diluted with 1 ml of H_2O and the $OD_{580\ nm}$ was determined. Values for bioassays using modified Starr's medium are from (33). Values for bioassays using nutrient broth are from the present study.

[b] Bacteria were grown in 0.22 ml of isoflavonoid-amended nutrient broth at 25 C at 210 strokes per minute on a reciprocal shaker for 21 hr. Treatments were diluted with 1 ml of H_2O and the $OD_{580\ nm}$ was determined. The $OD_{580\ nm}$ of the diluted control that had not been amended with isoflavonoids ranged from 0.116 to 0.263 (mean 0.218) for G32 and 0.172 to 0.329 (mean 0.280) for SR36. No correlation between the level of growth in the unamended medium controls and the activity of the isoflavonoids in the various trials was observed. The percentages of inhibition by dimethyl sulfoxide (DMSO) alone were 0 and 21% for G32 and SR36, respectively. The range of variation and the mean variation of individual DMSO control $OD_{580\ nm}$ values from the mean DMSO $OD_{580\ nm}$ were approximately ±10 and <6%, respectively.

[c] Negative percent inhibition represents stimulation relative to the mean $OD_{580\ nm}$ of DMSO controls.

TABLE 2. Phytoalexin accumulation in bean leaves infected by *Xanthomonas campestris* pv. *phaseoli*

Treatment				Phytoalexins recovered (μg/gm dry weight)[a]			
Cultivar	Inoculum conc. (CFU/ml)	Days after inoculation	Reaction	Phaseollin	Phaseollin-isoflavan	Coumestrol	Kievitone
Tara	10^8	2	HR	1,089	463	0	0[b]
Tara	10^8	4	HR	943	231[c]	167[c]	0
Tara	10^6	5	R	0	0	0	0
Tara	10^6	8	R	0[b]	0[b]	0	0
Dark Red Kidney	10^8	2 and 4	S	0	0	0	0
Dark Red Kidney	10^6	5 and 8	S	0	0	0	0

[a] One gram dry weight equals areas of 581 cm^2 for Tara and 559 cm^2 for Dark Red Kidney, respectively.

[b] Substances resembling the indicated isoflavonoid were detected but could not be positively identified as the known phytoalexin (see text).

[c] Observed isoflavonoid concentrations in this experiment were corrected for efficiency of extraction by mean efficiency values for the indicated compounds. In all other experiments the isoflavonoid concentrations were corrected for extraction efficiency by the efficiency of extraction of standard used in the same experiment. Efficiency ranges and means (in parentheses) were phaseollin 47–96% (74%), phaseollinisoflavan 42–88% (61%), kievitone 61–63% (62%), and coumestrol 69–88% (80%).

glycinea. In our work, however, the four bean isoflavonoid phytoalexins were not detectable in nonhypersensitively reacting Tara tissue 5 days after inoculation with 10^6 CFU per milliliter, when resistance had been established and *X. campestris* pv. *phaseoli* populations had ceased to increase. Even if bacteria are distributed nonuniformly as microcolonies of approximately 200 cells and only plant cells adjacent to microcolonies become necrotic in nonhypersensitively reacting resistant tissue (12,13), the number of host cells responding to the large *X. campestris* pv. *phaseoli* populations in the 581 cm^2 leaf per gram dry wt extracted should still have been significant. Given the amounts of tissue examined, the moderate to high efficiencies of isoflavonoid extraction (Table 2, footnote e), and the sensitivity of the tlc bioassay, the four phytoalexins should have been detectable in spite of possible localization if significant amounts (>7 μg/g dry wt) were present. If one assumes that the two inhibitory substances present at 8 days were impure forms of two of the isoflavonoids, they accumulated too late and in concentrations too low to inhibit the large *X. campestris* pv. *phaseoli* population already present at 5 days. The lack of detectable levels of the four isoflavonoids at early stationary phase and the apparently limited antibacterial activity of two of them therefore suggest that the characterized phytoalexins studied are unlikely to contribute significantly to the nonhypersensitive resistance of beans to *X. campestris* pv. *phaseoli*. These data, and those of Webster and Sequeira (32), moreover suggest that restricting investigations to hypersensitive tissues may result in misleading and possibly erroneous conclusions regarding the general involvement of known isoflavonoid phytoalexins in resistance.

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