Antibacterial Activity of Selected Isoflavonoids

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ABSTRACT

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Six isoflavonoids were screened in both semisolid agar and liquid media for antibacterial activity against 20 phytopathogenic and saprophytic isolates representing the genera *Pseudomonas*, *Xanthomonas*, and *Achromobacter*. Results of the two bioassay systems showed that coumestrol and formononetin lacked significant antibacterial activity. Phaseollin and pisatin were slightly to moderately inhibitory to a few isolates. Phaseollinisoflavan exhibited slight to moderate activity against several pseudomonads but strongly inhibited the xanthomonads and *Achromobacter* spp. Kievitone was the most inhibitory compound tested, significantly affecting the growth of a large proportion of the isolates. The xanthomonads and *Achromobacter* isolates tended to be more strongly inhibited by the active compounds than the pseudomonads. Preliminary results suggest that phaseollinisoflavan and kievitone are bactericidal to some isolates.

The accumulation of isoflavonoid phytoalexins in legumes in response to fungal infection is well documented (27). Although relatively little work on their occurrence in bacterial infections has been conducted, several isoflavonoids have been found to accumulate in French beans infected with *Pseudomonas phaseolicola* and soybeans infected with *P. glycinea* (7, 8, 9, 10, 12, 16, 17, 24). Moderately high amounts of the pterocarpans phaseollin and "hydroxyphaseollin" were present in extracts of hypersensitive-reacting bean and soybean tissues, respectively, about 72 hr after inoculation (10, 12, 24). ["Hydroxyphaseollin" is a mixture of three pterocarpan isomers now termed glyceollin I, II, and III (14).] The coumestan coumestrol accumulated in both plants, but the final levels attained were considerably lower than those of the pterocarpans (10, 12). Concentrations of phaseollin, "hydroxyphaseollin", and coumestrol in susceptible tissues were lower than those found in resistant reactions at a similar time after inoculation. The amount of the constitutive isoflavone daidzein increased in soybean in response to bacterial infection (10). The isoflavanone kievitone and the isoflavan phaseollinisoflavan also have been detected in halo-blighted bean leaves (17). Attempts to demonstrate bacterially-induced accumulation of pisatin in pea have been unsuccessful (5, 24).

Although the antifungal properties of several isoflavonoid phytoalexins have been demonstrated in a number of in vitro bioassays, the toxicity of these compounds to bacteria remains uncertain. Results of in vitro bioassays have varied widely among investigators (4, 6, 8, 9, 12, 24, 31). Only a limited number of isolates and a few compounds have been employed. Few studies have included bacterial species nonpathogenic on the plant used as a source of the compound under consideration (4, 6, 9, 12). This study was undertaken to determine whether six isoflavonoids are inhibitory in vitro to 20 plant pathogenic or saprophytic bacteria in two bioassay systems. The compounds used were selected to represent the five structural subclasses of isoflavonoids which accumulated in bacterially infected bean and soybean. Preliminary reports of the work have been made (32, 33).

MATERIALS AND METHODS

All bacterial isolates were kindly supplied by R. S. Dickey, Cornell University, with the following exceptions: *Achromobacter* sp., M. N. Schroth, University of California, Berkeley; *Pseudomonas phaseolicola* MeF and *Xanthomonas phaseoli* G32, R. E. Wilkinson, Cornell University; *P. aeruginosa*, T. L. Weaver, Cornell University; *P. allicola* 61-3 and *P. cepacia* 71-22, J. W. Lorbeer, Cornell University; *P. phaseolicola* G50 Tox^+ and G50 Tox^-, S. S. Patil, University of Hawaii; and *P. glycinea* race 1, B. W. Kennedy, University of Minnesota. *Pseudomonas* and *Achromobacter* isolates were maintained on nutrient agar [(NA), Difco Laboratories, Detroit, MI 48232] slants, whereas *Xanthomonas* isolates routinely were grown on potato-dextrose agar [(PDA), Difco]. Cultures were transferred to fresh media at monthly intervals and stored at 6 C. Purity was checked periodically by streaking dilute suspensions on NA and by observing colony morphology after 48 hr of incubation at 25 C.

Experiments to determine the virulence of the *P. phaseolicola* and *X. phaseoli* isolates were conducted after most bioassay experiments had been completed. All isolates tested were pathogenic on *Phaseolus vulgaris* L.

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'Dark Red Kidney' (Agway, Inc., Syracuse, NY 13201) when primary leaves were infiltrated with water suspensions of the bacteria. Virulence, however, varied widely among the isolates.

Phaseollin and phaseollinisoflavan were extracted from diseased beans (*Phaseolus vulgaris* L.) and pisatin was extracted from diseased pea (*Pisum sativum* L.) tissue and purified by methods described previously (21, 28, 29). Samples of the other compounds were kindly provided by the following investigators: kievitone, D. A. Smith, University of Hull, England; coumestrol, E. M. Bickoff, USDA-ARS Western Regional Research Laboratory, Berkeley, CA 94710; and formononetin, A. B. Beck, CSIRO, Rural Science Laboratory, South Perth, Western Australia, and S. Shibata, University of Tokyo, Japan. Compounds were dissolved in redistilled 95% ethanol or reagent grade dimethylsulfoxide (DMSO) and stored at −20 C. The concentrations of all compounds except coumestrol were determined from their ultraviolet absorbances and published extinction coefficients (19, 21, 23). Coumestrol solutions were prepared on a weight-volume basis.

The compounds initially were screened for antibacterial activity on a semisolid medium by a modification of the standard antibiotic-impregnated paper disk method. Bacterial cells were grown at 25 C for 48 hr on PDA in the case of xanthomonads and on NA for the remaining isolates. Cells were washed from slants with about 2 ml sterile H_2O, and 0.5 ml of the resultant turbid suspension was mixed rapidly with 4.5 ml of molten 0.7% (w/v) water agar (WA). The WA mixture was swirled rapidly over the surface of about 15 ml of NA + 1% glucose contained in 9-cm diameter petri plates. After the WA mixture had solidified (20 min), the plates were placed over a grid, and 1 μliter of isoflavonoid, dissolved in DMSO, was spotted directly on the surface of the semisolid WA layer with a 10-μliter syringe. The direct spotting technique was used because the low water solubility of the compounds employed limited their diffusion from paper disks saturated with the test solutions. Isoflavonoid concentrations used were: phaseollin 4.2 μg/μliter, pisatin 3.9 μg/μliter, phaseollinisoflavan 3.9 μg/μliter, kievitone 4.4 μg/μliter, coumestrol 3.7 μg/μliter, and formononetin 2.8 μg/μliter. At higher concentrations, formononetin precipitated on the agar surface. Each plate also was spotted with 1 μliter of DMSO alone as a solvent control. A positive check of 1 μliter of a 2.4 μg/μliter solution of chloramphenicol in DMSO also was included in each plate. Antibacterial activity was estimated after approximately 13 hr of incubation at 25 C by comparing turbidities in treated and untreated areas. Inhibition was rated on the following scale: 0 = no inhibition; 1 = a trace of activity; 2 = moderate activity or readily detectable spots in which the center of the treated zone was cloudy (Fig. 1-A); 3 = intermediate activity or reaction zones covered with only a trace of bacterial growth (Fig. 1-B); and 4 = a complete absence of growth (Fig. 1-C). Each compound was tested against each isolate a minimum of five times in two experiments.

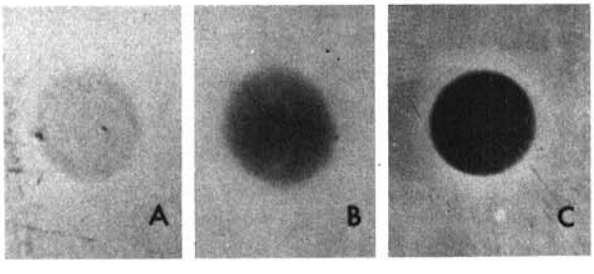

Fig. 1-(A to C). Typical zones of inhibition of bacteria by kievitone (4.4 μg/μliter) approximately 13 hr after 1 μliter of isoflavonoid solution was spotted on the surface of a semisolid agar layer containing the inoculum. **A)** Moderate inhibition (score 2) of *P. allicola* 61-3. **B)** Intermediate inhibition (score 3) of *P. tabaci* 11. **C)** Complete inhibition (score 4) of *Achromobacter* sp. isolate AB-4a.

To determine the minimum inhibitory dosages of each compound, each isolate was tested semiquantitatively against a range of isoflavonoid concentrations in a defined liquid medium. A modified version of Starr's medium (22) was made by dissolving 3.1 g $NaH_2PO_4 \cdot H_2O$, 3.9 g Na_2HPO_4, and 5.0 g NH_4Cl in 500 ml H_2O (solution A) and adding 5.0 g glucose, 0.2 g $MgSO_4 \cdot 7H_20$, 0.2 g methionine, 1.0 g glutamic acid, and Starr's microelements to a separate 500-ml volume of H_2O (solution B). The pH of each solution was adjusted to 6.9 - 7.1 with 1 N KOH or 1 N HCl. Equal volumes of solutions A and B were mixed after autoclaving separately at 1.05 kg/cm^2 for 15 min, and the pH was rechecked. Nutrient broth (Difco) was used for the isolates of *Achromobacter* sp. and *X. campestris*.

Cells grown on yeast-dextrose-calcium carbonate agar (YDC) slants (1) for about 24 hr at 25 C were suspended in approximately 4 ml of the medium. The concentration of bacterial cells was adjusted to give an optical density (OD) at 580 nm of 0.1 on a Bausch and Lomb Spectronic 20 colorimeter. Inoculum was prepared by adding 0.5 ml of the above suspension to 4.5 ml of sterile medium. While rapidly mixing the diluted suspension on a Vortex mixer, 0.22 ml was transferred with an automatic pipet to a 7×750 mm tube containing 4 μliters of DMSO or 4 μliters of isoflavonoid in DMSO. The contents of the tube were mixed immediately at high speed on a Vortex mixer. All compounds were tested at concentrations of 10, 30, 60, and 100 μM. In addition, phaseollin and coumestrol were tested at 140 μM and pisatin, phaseollinisoflavan, and kievitone were tested at 140 and 300 μM. Except for kievitone, maximum concentrations employed approached the limit of water solubility of the individual compounds in modified Starr's medium when the final concentration of DMSO was 1.8%. Similar volumes of inoculum were added to tubes lacking DMSO and served as medium-only controls. Tubes were placed in a reciprocal shaker incubator in a nearly horizontal position to maximize surface area for aeration and incubated at 25 C and 185 strokes/min. The pseudomonads were incubated for 15 hr with kievitone and for 12 hr with all other compounds. *Achromobacter* isolates were grown for 17 hr and the slower-growing xanthomonads were incubated with all compounds for 26 hr.

After incubation, samples were diluted with 1 ml of

H_2O and the turbidity was measured at 580 nm with a Perkin-Elmer Coleman 124 spectrophotometer. The percentage of inhibition was calculated from differences in OD of the isoflavonoid-treated sample and the mean OD_{580nm} of the DMSO controls. Inhibition by DMSO was similarly calculated from differences in the mean OD_{580nm} of DMSO controls and the mean OD_{580nm} of medium-only controls. The range of variation of OD of individual DMSO controls from the mean OD_{580nm} of DMSO controls was approximately ± 10%. The average percentage of variation of DMSO sample OD_{580nm} from the mean DMSO OD_{580nm} was generally 6% or less. Optical densities were used as a semiquantitative measure of bacterial growth because the large number of samples tested made plate counts impractical. Recent work (11) suggests that in the OD range between 0 and 0.2 there exists a direct relationship between OD values and bacterial dry weights. Preliminary experiments with some of the isolates employed also suggest that bacterial numbers determined by plate counts varied directly with OD. The mean OD_{580nm} for the diluted medium only controls ranged from 0.1 to 0.2 for *Achromobacter* and *Pseudomonas* isolates at 17 and 12 hr, respectively. After diluting with H_2O, *Xanthomonas* isolates had OD_{580nm} values of 0.05 to 0.09 at 26 hr.

Due to the limited quantities of the isoflavonoids that were available, each concentration of each compound was tested only once in each experiment in the liquid bioassay. Approximately 22 DMSO controls (about two controls per three isoflavonoid concentrations tested) were included in each experiment involving xanthomonad, *Achromobacter*, or *P. phaseolicola* isolates. Eleven DMSO controls were used in each experiment with the other isolates. All isolates of *P. phaseolicola* and *X. phaseoli* were tested with all six compounds in at least two experiments. The remaining isolates were tested once by the above methods, although the reaction of *P. tabaci* with phaseollinisoflavan was rechecked twice. The limited quantity of kievitone available precluded testing all isolates with that compound.

Additional tests of all compounds except kievitone also were done with all isolates except *X. phaseoli* G32 by a modification of the above method. Samples in these experiments were incubated in a vertical position without shaking for 18 hr at 25 C and turbidities of the undiluted samples were compared visually. Growth was rechecked at 48 and 72 hr. Samples lacking observable growth after 72 hr in the unshaken liquid assays were transferred to 5.5 ml of nutrient broth plus 1% glucose. Cultures were incubated on a reciprocal shaker at room temperature for 7 days and observed daily for growth. The effect of kievitone on bacterial survival was assessed by different methods. After the incubation periods normally employed in shake culture tests of kievitone, a loopful of any *P. tabaci* or *X. phaseoli* samples lacking detectable growth was streaked on NA. Numbers of colonies obtained after 48 and 96 hr at 25 C were compared with those observed on NA plates streaked with the inoculum at the start of the liquid assay. The effect of 300 μM phaseollinisoflavan on survival of *P. tabaci* 11 in 12-hr shake cultures was determined by the methods used for kievitone treatments.

RESULTS

In the semisolid medium bioassay chloramphenicol strongly inhibited (rating 3-4) all isolates except *P. allicola* and *P. aeruginosa*. The former isolate was moderately inhibited, whereas the latter was affected only slightly. The carrier solvent DMSO alone did not inhibit any of the isolates. Formononetin was ineffective against all isolates, and coumestrol exhibited only minimum activity against a single isolate (Table 1). The pterocarpans phaseollin and pisatin slightly inhibited the growth of a few isolates. Only the *Achromobacter* isolates were completely inhibited by phaseollin. Phaseollinisoflavan strongly inhibited the xanthomonads and the *Achromobacter* isolates and was slightly to moderately active against several of the pseudomonads. Kievitone was the most inhibitory compound tested, both in terms of the number of isolates affected and the degree of the antibacterial effect observed. Isolates of *Xanthomonas* and *Achromobacter* tended to be inhibited by the isoflavonoids more than were the pseudomonads.

Growth of all isolates at all concentrations of formononetin did not differ significantly from that in DMSO controls in liquid bioassays, and the data are therefore omitted from Table 2. Coumestrol also was noninhibitory to all isolates of *Pseudomonas* and *Xanthomonas*. Turbidities of *Achromobacter* AB-1a and AB-4a averaged approximately 30% and 40% of that in solvent controls, respectively, over the entire range of coumestrol concentrations tested. No clumping of cells was observed macroscopically to account for the decreased turbidity; prolonged mixing did not alter results. Phaseollin was slightly stimulatory to or inactive against all pseudomonads tested except *P. fluorescens*, which was strongly inhibited. Pisatin had a limited effect on most isolates of *Pseudomonas*. Phaseollinisoflavan was slightly to moderately active against several pseudomonad isolates and highly inhibitory to a few others. Kievitone at concentrations of 140 μM or greater significantly inhibited all the isolates tested. In general, isolates of *Xanthomonas* and *Achromobacter* were more sensitive to the four compounds listed in Table 2 than the pseudomonads. Inhibition by DMSO alone was observed in the liquid bioassays, but the level varied widely among the isolates. No correlation between inhibition by DMSO and sensitivity to the test phytoalexins was observed.

The pattern of sensitivity of the various isolates to the isoflavonoids generally was similar in both bioassay systems, but some discrepancies were noted. *Pseudomonas pisi* 6 apparently was more tolerant of pisatin, phaseollinisoflavan, and kievitone in liquid medium than in the semisolid agar. In contrast, *X. phaseoli* 34 and *X. vesicatoria* 21 were more sensitive to phaseollin and pisatin, respectively, in the liquid bioassays than in the semisolid medium bioassay.

Preliminary tests indicated that a 72-hr exposure of *X. phaseoli* 34, *X. vesicatoria* 21, and *X. campestris* 3 to 300 μM phaseollinisoflavan completely prevented subsequent growth when the isoflavonoid-treated cultures were diluted 27.5-fold with fresh medium. A similar period of exposure of *Achromobacter* AB-1a and AB-4a to 60 μM phaseollinisoflavan also prevented growth in diluted cultures. Markedly fewer colonies of *P. tabaci* 11 developed on plates streaked with a loopful of

12-hr shake cultures containing 300 μM phaseollinisoflavan than on plates streaked with the initial inoculum. Kievitone (60 μM) greately reduced the number of colonies of *X. phaseoli* 34, *X. phaseoli* G32, and *P. tabaci* 11 recovered, compared to those in the initial inoculum. No colonies of either xanthomonad developed on plates streaked with samples from treatments containing higher kievitone concentrations. The number of *P. tabaci* colonies recovered from kievitone-treated cultures decreased progressively as isoflavonoid concentration increased; at 140 and 300 μM no growth was observed.

DISCUSSION

These results indicated that four of the six isoflavonoids tested possess some antibacterial properties. In contrast to previously published reports (8, 9, 10, 12), coumestrol lacked significant antibacterial activity in the semisolid and liquid media bioassays, although some cellular clumping occasionally was observed in the latter. The limited inhibitory effects of coumestrol and formononetin on bacteria is similar to their reported slight activity against fungi (3, 18, 26, 30). Although the four remaining compounds strongly inhibited some isolates, the observed antibacterial activity generally was less than their reported antifungal activity (4, 6, 21, 27). The slight-to-moderate activity of the pterocarpans phaseollin and pisatin against most of the bacterial isolates tested contrasts sharply with the marked inhibition of many fungi by both compounds at the maximum concentrations employed in this study (4, 6).

The sensitivity of the bacterial isolates to the compounds tested varied widely. The pseudomonads generally were more tolerant of the isoflavonoids employed than the xanthomonads or *Achromobacter* isolates. The apparent greater sensitivity of the xanthomonads to several of the compounds cannot be attributed solely to their poor growth in the liquid assays, since comparable high levels of inhibition were detected in the bioassays on semisolid media, in which their growth was excellent. No clear correlation between pathogenicity on leguminous species and tolerance to the isoflavonoids was evident. The reaction of the *P. phaseolicola* isolates could not be correlated with race. *Pseudomonas phaseolicola* 7 was classified as race 1 by Patel and Walker (15) on the basis of foliar symptoms, but behaved similarly to the race 2 isolates G50 Tox$^+$ and G50 Tox$^-$ (8). The kievitone ED_{50} values for the five isolates of *P. phaseolicola* and the different degrees of inhibition of the two *X. phaseoli* isolates by several compounds suggest that reaction to a given isoflavonoid is isolate-dependent within a nomenspecies.

The absence of antibacterial activity of several of the compounds in these studies does not appear to result solely from the bioassay conditions employed. Chloramphenical, which is a known antibacterial compound, stronly inhibited the growth of all except two isolates in the bioassays on semisolid media. More importantly, some isoflavonoids (e.g., kievitone) had significant activity in both types of bioassays. Although fairly high inoculum levels (about 10^7 cells/ml) were used to facilitate turbidimetric measurements after relatively short incubation periods, inoculum density alone seems inadequate to explain the low activity that was observed.

TABLE 1. Effect of six isoflavonoids on bacteria in the semisolid medium bioassay

Isolate[c]	Degree of inhibition[a, b]					
	Phaseollin	Pisatin	Phaseollin-isoflavan	Kievitone	Coumestrol	Formononetin
P. phaseolicola 7	0.8	2	1	2	0	0
P. phaseolicola G50 Tox$^+$	0	0	0	2	0	0
P. phaseolicola G50 Tox$^-$	0	0	0	2	0	0
P. glycinea race 1	0	0	0	0	0	0
P. pisi 6	0	0.4	2.3	3.3	0	0
P. tabaci 11	0	0	2	3	0	0
P. angulata 12	0	0	0.8	2	0	0
P. allicola 61-3	0	0	0.5	2	0	0
P. cepacia 71-22	0	0	1	2	0	0
P. fluorescens SR	1.8	1.6	3.6	4	0	0
P. aeruginosa	0	0	0	0	0	0
X. phaseoli 34	0.4	0	4	3.6	0	0
X. phaseoli G32	1.3	0	3.5	4	0	0
X. vesicatoria 21	1.5	1	4	4	0	0
X. campestris 3	0.9	0.4	4	4	0	0
Achromobacter sp.						
AB-1a	4	0	4	4	0	0
AB-4a	4	0	4	4	0.7	0

[a] One μliter of isoflavonoid dissolved in DMSO was spotted on the surface of a semisolid agar layer in which the inoculum was uniformly mixed. Concentrations employed were: phaseollin 4.2 μg/μliter; pisatin 3.9 μg/μliter; phaseollinisoflavan 3.9 μg/μliter; kievitone 4.4 μg/μliter; coumestrol 3.7 μg/μliter; and formononetin 2.8 μg/μliter.

[b] Rating scale: 0 = no inhibition; 1 = trace; 2 = moderate; 3 = intermediate; 4 = complete inhibition. Scores are the mean of ratings of at least 5 replicates.

[c] Bacterial isolates: *Pseudomonas*, *Xanthomonas*, and *Achromobacter* spp.

TABLE 2. The effect of four isoflavonoids on growth of bacteria in liquid media[a]

Isolate	Inhibition of growth relative to DMSO controls								
	Phaseollin		Pisatin		Phaseollinisoflavan		Kievitone		
	ED_{50} (μM)	% Inhibition @ maximum conc. tested (140 μM)	ED_{50} (μM)	% Inhibition @ maximum conc. tested (300 μM)	ED_{50} (μM)	% Inhibition @ maximum conc. tested (300 μM)	ED_{50} (μM)	% Inhibition @ maximum conc. tested (300 μM)	(%) Inhibition by DMSO
P. phaseolicola 7	>140	−7[d]	>300	7	>300	23	~140	96	27
P. phaseolicola 14	>140	−12	>300	12	>300	22	~100	96	23
P. phaseolicola MeF	>140	−7	>300	6	>300	11	100-140	98	35
P. phaseolicola G50 Tox	>140	−8	>300	−4	>300	27	140	93	34
P. phaseolicola G50 Tox	>140	−6	>300	20	>300	42	60-100	100	34
P. glycinea race 1	>140	−1	>300	−7	>300	12	140	93	17
P. pisi 6	>140	−8	>300	2	>300	17	140-300	80	3
P. tabaci 11	>140	−5	>300	21	60-100	99	30-60	100	46
P. angulata 12	>140	−12	>300	38	~140	63			13
P. lachrymans 12	>140	6	>300	9	>300	19			11
P. allicola 61-3	>140	4	>300	−25	>300	39			42
P. cepacia 71-22	>140	10	>300	5	140-300	56			24
P. fluorescens SR	~30[b]	100[b]	~100[b]	100[b]	~30[b]	100[b]			ND[b]
P. aeruginosa	>140[b]	0[b]	>300[b]	0[b]	>300[b]	0[b]			ND[b]
X. phaseoli 34	60	89	140-300	99	10-30	100	10-30	99	−6
X. phaseoli G32	>140	22	~300	43	30-60	100	10-30	99	14
X. vesicatoria 21	30-60[c]	78	140-300	90	10-30	100			9
X. campestris 3	~60[b]	30[b]	~300[b]	50[b]	30-60[b]	100[b]			6
Achromobacter sp.									
AB-1a	10-30[b]	100[b]	100[b]	100[b]	10-30[b]	100[b]			ND[b]
AB-4a	10-30	87	140	90	10-30	97			17

[a]Bacteria were grown in 0.22 ml of isoflavonoid-amended liquid medium at 25 C at 185 strokes/min on a reciprocal shaker for periods indicated in the text. At the conclusion of the incubation period, treatments were diluted with 1 ml H_2O and the $OD_{580\ nm}$ was determined.

[b]Estimated visually due to bacterial clumping. Visual estimates of growth of AB-1a were based on still culture assays in which clumping was not observed. Percent inhibition by DMSO was not determined (ND).

[c]The ED_{50} value was between the two concentrations stated.

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

Phaseollin exhibited little or no inhibitory activity against phytopathogenic pseudomonads at inoculum levels of 10^3 cells/ml (12), 10^5 cells/ml (31), or bacterial numbers similar to those used in this work (6, 24). The level of inhibition of fungi in vitro by isoflavonoid phytoalexins has been shown to be influenced by the chemical and physical properties of the medium (20, 25), age of the inoclum (2), and the growth stage of the organism population being tested (25). Therefore, differences in bioassay methods may account for discrepancies in the literature regarding the antibacterial activity of some of the compounds (8, 9, 12). However, when uniform bioassay conditions were employed in tests of individual isolates, none of the 20 isolates was inhibited by all six of the compounds in either bioassay system, and results of the two bioassays corresponded well (Tables 1 and 2). The variation in reaction to the isoflavonoids thus appears to reflect differences in the antibacterial activity of the compounds and in intrinsic sensitivity of the isolates to the various compounds.

One bioassay parameter which may critically affect the degree of phytoalexin activity observed is the length of the incubation period. In initial liquid still-culture bioassays in which bacterial multiplication was assessed visually, minimum inhibitory dosages of several compounds were higher at 72 hr than at 18 or 48 hr (J. G. Wyman, *unpublished*). Assessments of growth after short incubation periods may suggest exaggerated effects of the compounds on test microorganisms, but these are useful preliminary screens for detecting active compounds which may warrant more detailed study. Determinations of growth after prolonged incubation periods may fail to detect phytoalexin-associated differences in lag phases or growth rates (24,31).

Because the in vitro and in vivo environments differ, the degree to which in vitro bioassay toxicity data can be extrapolated to the host-pathogen interaction is limited. Although most of the bacterial isolates pathogenic on legumes were quite tolerant of the isoflavonoids, some isolates were very sensitive to several of the compounds produced by the plant species with which they are normally associated. The *Achromobacter* isolates, which have been reported to enhance halo blight severity in beans (13), and *X. phaseoli* both were strongly inhibited by several of the bean phytoalexins. In spite of their sensitivity to isoflavonoids in vitro, both *X. phaseoli* isolates caused large, spreading necrotic lesions in inoculated beans. A determination of whether these compounds are inhibitory to either of these bacterial species in vivo awaits the results of further research.

LITERATURE CITED

1. BAIGENT, N. L., J. E. DE VAY, and M. P. STARR. 1963. Bacteriophages of Pseudomonas syringae. N. Z. J. Sci. 6:75-100.
2. BAILEY, J. A., G. A. CARTER, and R. A. SKIPP. 1976. The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiol. Plant Pathol. 8:189-194.
3. BICKOFF, E. M., R. R. SPENCER, S. C. WITT, and B. E. KNUCKLES. 1969. Studies on the chemical and biological properties of coumestrol and related compounds. U.S. Dep. Agric. Tech. Bull. 1408. 95 p.
4. CRUICKSHANK, I. A. M. 1962. Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. Aust. J. Biol. Sci. 15:147-159.
5. CRUICKSHANK, I. A. M., and D. R. PERRIN. 1963. Studies on phytoalexins. VI. Pisatin: The effect of some factors on its formation in Pisum sativum L., and the significance of pisatin in disease resistance. Aust. J. Biol. Sci. 16:111-128.
6. CRUICKSHANK, I. A. M., and D. R. PERRIN. 1971. Studies on phytoalexins. XI. The induction, antimicrobial spectrum and chemical assay of phaseollin. Phytopathol. Z. 70:209-229.
7. GNANAMANICKAM, S., and S. S. PATIL. 1975. Induction of phytoalexins in bean by Pseudomonas phaseolicola. Proc. Am. Phytopathol. Soc. 2:64 (Abstr.).
8. GNANAMANICKAM, S. and S. S. PATIL. 1976. Antibacterial activity of phytoalexins induced by Pseudomonas phaseolicola in resistant bean leaves. Proc. Am. Phytopathol. Soc. 3:261 (Abstr.).
9. GNANAMANICKAM, S. S., and S. S. PATIL. 1977. Accumulation of antibacterial isoflavonoids in hypersensitively responding bean leaf tissues inoculated with Pseudomonas phaseolicola. Physiol. Plant Pathol. 10:159-168.
10. KEEN, N. T., and B. W. KENNEDY. 1974. Hydroxyphaseollin and related isoflavonoids in the hypersensitive resistant response of soybean against Pseudomonas glycinea. Physiol. Plant Pathol. 4:173-185.
11. LAWRENCE, J. V., and S. MAIER. 1977. Correction for the inherent error in optical density readings. Appl. Environ. Microbiol. 33:482-484.
12. LYON, F. M., and R. K. S. WOOD. 1975. Production of phaseollin, coumestrol and related compounds in bean leaves inoculated with Pseudomonas spp. Physiol. Plant Pathol. 6:117-124.
13. MAINO, A. L., M. N. SCHROTH, and V. B. VITANZA. 1974. Synergy between Achromobacter sp. and Pseudomonas phaseolicola resulting in increased disease. Phytopathology 64:277-283.
14. PARTRIDGE, J. E., and N. T. KEEN. 1977. Soybean phytoalexins: rates of synthesis are not regulated by activation of initial enzymes in flavonoid biosynthesis. Phytopathology 67:50-55.
15. PATEL, P. N., and J. C. WALKER. 1965. Resistance in Phaseolus to halo blight. Phytopathology 55:889-894.
16. PATIL, S. S., and S. GNANAMANICKAM. 1975. Phaseotoxin causes suppression of Pseudomonas phaseolicola induced hypersensitive response and phytoalexin accumulation in resistant bean leaves. Proc. Am. Phytopathol. Soc. 2:64 (Abstr.).
17. PATIL, S. S., and S. S. GNANAMANICKAM. 1976. Suppression of bacterially-induced hypersensitive reaction and phytoalexin accumulation in bean by phaseotoxin. Nature (Lond.) 259:486-487.
18. PERRIN, D. R., and I. A. M. CRUICKSHANK. 1969. The antifungal activity of pterocarpans towards Monilinia fructicola. Phytochemistry 8:971-978.
19. PUEPPKE, S. G., and H. D. VANETTEN. 1974. Pisatin accumulation and lesion development in peas infected with Aphanomyces euteiches, Fusarium solani f. sp. pisi, or Rhizoctonia solani. Phytopathology 64:1433-1440.
20. PUEPPKE, S. G., and H. D. VANETTEN. 1976. The relation between pisatin and the development of Aphanomyces euteiches in diseased Pisum sativum. Phytopathology 66:1174-1185.
21. SMITH, D. A., H. D. VANETTEN, and D. F. BATEMAN. 1975. Accumulation of phytoalexins in Phaseolus vulgaris hypocotyls following infection by Rhizoctonia solani. Physiol. Plant Pathol. 5:51-64.
22. STARR, M. P. 1946. The nutrition of phytopathogenic

bacteria: I. Minimal nutritive requirements of the genus Xanthomonas. J. Bacteriol. 51:131-143.
23. STECHER, P. G., M. WINDHOLZ, D. S. LEAHY, D. M. BOLTON, and L. G. EATON (eds.). 1968. The Merck Index (Eighth Ed.). Merck & Co., Inc., Rahway, New Jersey. 1713 p.
24. STHOLASUTA, P., J. A. BAILEY, V. SEVERIN, and B. J. DEVERALL. 1971. Effect of bacterial inoculation of bean and pea leaves on the accumulation of phaseollin and pisatin. Physiol. Plant Pathol. 1:177-183.
25. VANETTEN, H. D. 1973. Differential sensitivity of fungi to pisatin and to phaseollin. Phytopathology 63:1477-1482.
26. VANETTEN, H. D. 1976. Antifungal activity of pterocarpans and other selected isoflavonoids. Phytochemistry 15:655-659.
27. VANETTEN, H. D., and S. G. PUEPPKE. 1976. Isoflavonoid phytoalexins. Pages 239-289 *in* J. Friend and D. R. Threlfall, eds., Biochemical aspects of plant-parasitic relationships. Annu. Proc. Phytochem. Soc. Vol. 13. Academic Press, London. 354 p.
28. VANETTEN, H. D., S. G. PUEPPKE, and T. C. KELSEY. 1975. 3, 6a-dihydroxy-8,9-methylenedioxypterocarpan as a metabolite of pisatin produced by Fusarium solani f. sp. pisi. Phytochemistry 14:1103-1105.
29. VANETTEN, H. D., and D. A. SMITH. 1975. Accumulation of antifungal isoflavonoids and la-hydroxyphaseollone, a phaseollin metabolite, in bean tissue infected with Fusarium solani f. sp. phaseoli. Physiol. Plant Pathol. 5:225-237.
30. VIRTANEN, A. I., and P. K. HIETALA. 1958. Isolation of an anti-Sclerotinia factor, 7-hydroxy-4′-methoxyisoflavone from red clover. Acta Chem. Scand. 12:579-580.
31. WEBSTER, D. M., and L. SEQUEIRA. 1977. Expression of resistance in bean pods to an incompatible isolate of Pseudomonas syringae. Can. J. Bot. 55:2043-2052.
32. WYMAN, J. G., and H. D. VANETTEN. 1975. The antibacterial activity of six isoflavonoid phytoalexins. Proc. Am. Phytopathol. Soc. 2:110 (Abstr.).
33. WYMAN, J. G., and H. D. VANETTEN. 1976. In vitro effects of five isoflavonoid phytoalexins against Pseudomonas phaseolicola. Proc. Am. Phytopathol. Soc. 3:262 (Abstr.).