

Selective Toxicity of Isoflavonoid Phytoalexins to Gram-Positive Bacteria

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Accepted for publication 6 March 1980.

ABSTRACT

GNANAMANICKAM, S. S., and D. A. SMITH. 1980. Selective toxicity of isoflavonoid phytoalexins to Gram-positive bacteria. *Phytopathology* 70:894-896.

The isoflavonoid phytoalexins, kievitone and phaseollin, were found to be selectively toxic to Gram-positive bacteria. In a standard paper-disk bioassay, 10–50 µg kievitone or phaseollin inhibited the growth of all seven Gram-positive, but none of the eight Gram-negative, bacteria tested. Phaseollidin and phaseollinisoflavan also possessed such selective toxicity

Additional key word: antibiotic.

to Gram-positive bacteria. Further assays showed that even 2 µg kievitone (0.56×10^{-8} mole), which proved to be the most toxic of the compounds examined, inhibited the growth of *Corynebacterium fascians*, *Bacillus subtilis*, and *Micrococcus luteus*.

Phytoalexins have been defined as antibiotics that are produced by interacting plant host and parasite metabolic systems, and inhibit the growth of microorganisms pathogenic to plants (9). Most research into phytoalexins has established that these compounds are toxic to fungi (4,12,17) and it has been hypothesized that they play an important role in disease resistance in some plants (4).

Several of the isoflavonoid phytoalexins (17) also have been found in association with the hypersensitive response (HR) exhibited by plants in response to bacterial invasions (6–8,10,15). Results of in vitro bioassays showed that phytoalexins of soybean

and French bean were toxic to *Pseudomonas glycinea* and *P. phaseolicola* (6–8). However, the results from a similar study made by Wyman and VanEtten (19) did not entirely confirm the above findings. Therefore, the effects of isoflavonoids against plant pathogenic bacteria remain uncertain.

In the present study, we examined the sensitivity of several species of plant pathogenic and saprophytic bacteria to isoflavonoid phytoalexins from French bean. Our results suggested that isoflavonoids are toxic to Gram-positive, but not Gram-negative, bacteria.

MATERIALS AND METHODS

Bacterial strains. The 15 species of bacteria (eight Gram-negative and seven Gram-positive) used in this study are described in Table 1.

Antibiotic assay. A paper-disk method was used. The antibiotic assay disks (Whatman A. A. disks, 0.6 cm diameter) were loaded with a prescribed amount of the phytoalexin in absolute ethanol (maximum volume, 25 μ l). In each plate, there was a control disk treated with 25 μ l of ethanol alone. In all cases, sufficient time was allowed for the ethanol to evaporate before transferring the disks to agar surfaces. The disks were arranged in petri dishes containing a bottom layer of nutrient agar (Oxoid) and a top layer of soft agar (nutrient agar with 0.75% agar) seeded with the test bacterium. Each bacterial strain was grown at 30 C for 16 hr in nutrient broth. One hundred microliters of the culture ($\sim 10^8$ cells per milliliter) were added to 2 ml of molten soft agar (45 C), mixed thoroughly and poured over the nutrient agar surface. Plates were incubated at 30 C and diameters of inhibition zones were measured after 20 hr.

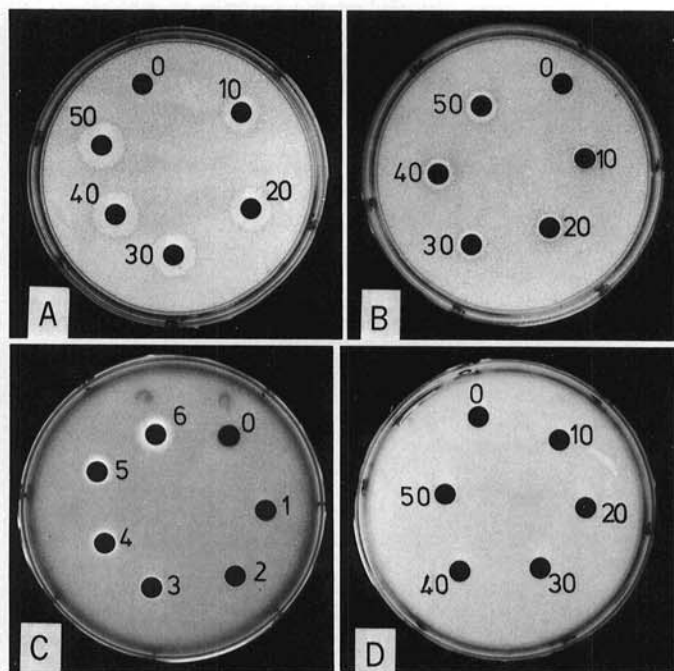


Fig. 1. Inhibition of growth of *C. fascians* by **A**, kievitone or **B**, phaseollin at 10–50 μ g/disk and of *M. luteus* by **C**, kievitone at 1–6 μ g/disk. Lack of growth inhibition in *X. campestris* by **D**, kievitone at 10–50 μ g/disk.

Results in Table 1 represent mean values of two separate experiments.

Isoflavonoid phytoalexins. Phaseollin (a pterocarpan) and kievitone (an isoflavanone) used in this study were extracted from bean hypocotyl lesions infected with *Rhizoctonia solani* Kühn, or rotting cowpea seeds, as described elsewhere (12,14). These compounds were used in preliminary assays at 50, 100, and 200 μ g/disk and subsequently at 10, 20, 30, 40, and 50 μ g/disk. These latter amounts represent 2.8×10^{-8} , 5.6×10^{-8} , 8.4×10^{-8} , 1.1×10^{-7} , and 1.4×10^{-7} mole of kievitone per disk and 8.1×10^{-8} , 6.2×10^{-8} , 9.3×10^{-8} , 1.2×10^{-7} , and 1.6×10^{-7} mole of phaseollin per disk. Two more isoflavonoid phytoalexins, phaseollidin (a pterocarpan) and phaseollinisoflavan (an isoflavan) also were included in one set of assays against three Gram-positive and three Gram-negative bacteria. These compounds were tested at 25 and 50 μ g/disk. Phaseollinisoflavan, like phaseollin, was isolated from *R. solani*-infected beans. Phaseollidin was the gift of J. A. Bailey, Long Ashton Research Station, Bristol, U.K.; this material was subjected to one additional thin-layer chromatographic step before use.

RESULTS

Kievitone and phaseollin inhibited all seven Gram-positive bacteria and none of the eight Gram-negative bacteria. The Gram-positive bacteria gave distinct inhibition zones at 10, 30, and 50 μ g kievitone and phaseollin (Table 1). Kievitone induced the largest inhibition zones in plates seeded with sensitive bacteria. For example, the areas of kievitone-induced inhibition with *Corynebacterium fascians* were two to three times larger than those induced by phaseollin at the same concentrations (Table 1). In comparable assays, the Gram-negative bacteria generally were insensitive (Table 1). Occasionally, faint areas of inhibition seemed to develop in plates with *Erwinia carotovora*, but these were not consistent and the zones were never clear and consequently were not measured. Examples of typical assays are shown in Fig. 1.

In the preliminary assays with kievitone and phaseollin at 50, 100, and 200 μ g, the Gram-negative plant pathogens, *P. phaseolicola* (race 1 and 2), *Xanthomonas campestris*, *Agrobacterium tumefaciens*, and *E. carotovora* were not inhibited. On the other hand, less than 6 μ g kievitone and phaseollin inhibited the Gram-positive bacteria, *C. fascians*, *Bacillus subtilis*, and *Micrococcus luteus*. In two separate experiments, *C. fascians* was inhibited by 2 μ g (0.56×10^{-8} mole) of kievitone and the inhibition zone measured 25 mm². Phaseollin was toxic to *C. fascians* at 3 μ g

TABLE 1. Differential response of Gram-negative and Gram-positive bacteria to kievitone and phaseollin

Gram reaction and bacterial isolate	Source	Area of inhibition (mm ²) ^a					
		Kievitone (μ g/disk)			Phaseollin (μ g/disk)		
		10	30	50	10	30	50
Gram-negative:							
<i>Erwinia carotovora</i> var. <i>carotovora</i>	468 (NCPBP) ^b	0	0	0	0	0	0
<i>E. carotovora</i> var. <i>atroseptica</i>	549 (NCPBP)	0	0	0	0	0	0
<i>Pseudomonas phaseolicola</i> , race 1	1098 (NCPBP)	0	0	0	0	0	0
<i>P. phaseolicola</i> , race 2	882 ^c	0	0	0	0	0	0
<i>Agrobacterium radiobacter</i> var. <i>tumefaciens</i>	2270 (NCPBP)	0	0	0	0	0	0
<i>Escherichia coli</i>	B6 (HCC) ^d	0	0	0	0	0	0
<i>Salmonella typhimurium</i>	HU 103 (HCC)	0	0	0	0	0	0
<i>Xanthomonas campestris</i>	529 (NCPBP)	0	0	0	0	0	0
Gram-positive:							
<i>Corynebacterium fascians</i>	1675 (NCPBP)	137	289	337	59	95	126
<i>Bacillus subtilis</i>	1246 (NCPBP)	50	90	106	8	32	39
<i>B. megaterium</i>	B2 (HCC)	48	85	101	8	22	35
<i>Micrococcus luteus</i>	B15 (HCC)	115	193	227	5	29	32
<i>M. roseus</i>	B7 (HCC)	79	180	353	16	29	32
<i>Streptomyces griseus</i>	B21 (HCC)	85	143	161	10	39	63
<i>Mycobacterium phlei</i>	B8 (HCC)	76	129	144	38	53	60

^a Area of inhibition = (area of total inhibition - area of disk); each value is the mean of duplicate values rounded off to the nearest whole number. Assay disks were placed on the surface of petri dishes with a bottom layer of nutrient agar and an upper layer of soft (0.75%) nutrient agar seeded with the test bacterium.

^b NCPBP = National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, U.K.

^c J. D. Taylor, National Vegetable Research Station, Wellesbourne, U.K.

^d HCC = Culture Collection at the Department of Plant Biology, The University, Hull, HU6 7RX, U.K.

(0.93×10^{-8} mole), but the area of inhibition was only 10 mm^2 . At $2 \mu\text{g}$ kievitone and $4 \mu\text{g}$ phaseollin, growth of *B. subtilis* and *M. luteus* was inhibited (Fig. 1C).

Phaseollinisoflavan and phaseollidin, each at 25 and $50 \mu\text{g}$, were also toxic to the growth of the Gram-positive bacteria assayed, *C. fascians*, *B. subtilis*, and *M. luteus*. The Gram-negative bacteria tested, *P. phaseolicola* (race 1), *X. campestris* and *Salmonella typhimurium*, were not inhibited.

Control disks were not inhibitory to the growth of any of the 15 species of bacteria.

DISCUSSION

The results of this study indicate that Gram-positive bacteria are quite sensitive to isoflavonoids. This was emphasized by as little as $2 \mu\text{g}$ kievitone inhibiting the growth of Gram-positive bacteria (Fig. 1C) whereas levels as high as $200 \mu\text{g}$ did not prevent the growth of Gram-negative bacteria. This is the first report that isoflavonoids are selectively toxic to Gram-positive bacteria.

Although previous research on antibacterial assays of isoflavonoids does not show such a clear-cut distinction (1-3,8,16), it is nonetheless evident that Gram-positive bacteria proved to be more sensitive. *Brevibacterium linens*, a Gram-positive species, was inhibited by six fractions from bean leaf extracts (8). Only three of these were found to be active against *P. phaseolicola*: phaseollin and coumestrol were two of the three fractions. Albersheim and Valent (1) showed that *B. subtilis* was more inhibited by glyceollin than *P. glycinea*.

The comparative insensitivity of Gram-negative species to isoflavonoids was evidenced by Wyman and VanEtten (18,19). However, inconsistencies in the literature make generalizations difficult. Whereas Wyman and VanEtten found relatively little activity against *P. phaseolicola*, others have reported that isoflavonoids are toxic to this bacterium (6,10). The principal exception to Gram-negative insensitivity appears to be the genus *Rhizobium* (1,2).

Although procedural and isolate differences might explain some of the variable results with the isoflavonoids, this cannot always be the case (cf *Mycobacterium phlei* [2,3]). Because of the inconsistent findings reported, it seems essential that the antibacterial activities of isoflavonoid phytoalexins be re-examined and we hope that the present report might act as the necessary stimulus.

It is perfectly reasonable that isoflavonoids should be more effective against Gram-positive bacteria; antibacterial antibiotics, in general, are more toxic to Gram-positive bacteria. The exceptions, of course, are some of the well known broad-spectrum antibiotics which affect both Gram-positive and Gram-negative bacteria. The antibiotics which are more toxic to Gram-positive bacteria include the actinomycins, a few of the polymixins, micromonosporin, micrococin, and others (5). The differences in cell wall complexities may account for the greater sensitivity of the Gram-positive, and less or lack of sensitivity of the Gram-negative, bacteria. Alternatively, the Gram-positive bacteria may possess sensitive membrane sites where isoflavonoids bind irreversibly. It has been hypothesized that kievitone is membranolytic on *R. solani*, a fungal pathogen of bean (13).

The rapid and sensitive method of assay employed in the present study upholds the view (2,3,18,19) that Gram-negative phytopathogenic bacteria are not very sensitive to isoflavonoid phytoalexins. From our selection of bacteria, however, it is not possible to say whether there is a causal relationship between insensitivity to isoflavonoid phytoalexins and pathogenicity towards plants.

The toxicity of isoflavonoid phytoalexins to Gram-positive bacteria could make them a useful group of compounds in a

practical sense. It is conceivable that plant diseases caused by Gram-positive *Corynebacterium* spp. could be controlled by isoflavonoid treatments, especially with kievitone which is the most toxic of the four compounds tested. The fact that the sensitive *C. fascians* is very closely related (serologically) to corynebacteria responsible for human and animal diseases (11), and again, the sensitivity of *M. phlei*, suggest a possible medical value for the isoflavonoids.

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