Inhibition of Bacteria by the Soybean Isoflavonoids Glyceollin and Coumestrol

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Reference to brand or firm name does not constitute endorsement by the USDA over others of a similar nature not mentioned.
We thank John Phillips for conducting the statistical analyses.
Accepted for publication 20 October 1981.

ABSTRACT

Fett, W. F., and Osman, S. F. 1982. Inhibition of bacteria by the soybean isoflavonoids glyceollin and coumestrol. Phytopathology 72:755-760.

The soybean isoflavonoids glyceollin and coumestrol were tested for inhibitory activity against various strains of bacteria including the soybean bacterial leaf pathogens *Pseudomonas syringae* pv. *glycinea* and *Xanthomonas campestris* pv. *glycines*. Six different bioassays were used to test for effects of glyceollin and two for coumestrol. Glyceollin inhibited growth of most bacteria, including *P. syringae* pv. *glycinea* and *X. campestris* pv. *glycines*, and was shown to be bactericidal towards strains of *Bacillus subtilis*, *B. licheniformis*, *Staphylococcus aureus*, and *Corynebacterium flaccumfaciens* pv. *flaccumfaciens*. Three bioassays with agar media were evaluated; direct spotting onto agar media seeded with bacteria was the most efficient method for demonstrating the inhibitory activity of glyceollin. Only bioassays in liquid media demonstrated inhibitory activity of glyceollin against *P. syringae* pv. *glycinea*. Two strains of *P. syringae* pv. *glycinea* were shown to differ markedly in sensitivity to glyceollin. Coumestrol was bactericidal towards strains of *B. subtilis*, *B. licheniformis*, *S. aureus*, *Streptococcus thermophilus*, and *X. campestris* pv. *glycines* but did not inhibit growth of four *P. syringae* pv. *glycinea* strains. The results indicate a possible role of glyceollin and coumestrol in inhibiting the bacterial pathogen *X. campestris* pv. *glycines* and secondary bacterial invaders.

Bacteria are known to elicit phytoalexin production by plants (8,12,16), but the role of phytoalexins in host resistance is still unclear. Even the antibacterial activity of a single phytoalexin is subject to conflicting results. Several researchers (4,16,25,26) reported that phaseollin, the bean (*Phaseolus vulgaris* L.) pterocarpanoid phytoalexin, was not inhibitory against *Pseudomonas syringae* pv. *phaseolicola*, although this bacterium could elicit phaseollin production. Gnanamanickam and Patil (8), however, reported phaseollin to be bactericidal towards *P. syringae* pv. *phaseolicola*. More recently, Gnanamanickam and Smith (9) found no activity of phaseollin against this leaf pathogen by use of a filter paper disk bioassay.

The possible reasons for such conflicting results are numerous. It is well documented that the results of in vitro bioassays for phytoalexin antifungal activity depend on media composition (20), bioassay used (23), and growth stage of the fungus (21,23). Results of bioassays for antibacterial activity can be affected by media pH and composition, the bioassay used, inoculum size, incubation time and temperature, and subjectivity in reading results (5,17,18,24).

In initial attempts to obtain mutants of *P. syringae* pv. *glycinea* (causal agent of bacterial blight of soybean (*Glycine max* (L.) Merr. [22]) resistant to the reported bacteriostatic effects of glyceollin, the soybean pterocarpanoid phytoalexin (1,12), we could not demonstrate inhibitory activity of glyceollin against *P. syringae* pv. *glycinea* with an agar-incorporation bioassay. Consequently, we have reexamined the activity of glyceollin against *P. syringae* pv. *glycinea*, *Xanthomonas campestris* pv. *glycines* (causal agent of bacterial pustule disease of soybean [22]) and other bacteria. Investigators previously reporting on the antibacterial activity of phytoalexins have usually employed only one or two bioassays. We used up to six different bioassays and compared the results.

MATERIALS AND METHODS

Cultures. *Pseudomonas syringae* pv. *phaseolicola* strain 1134 (until recently thought to be *P. syringae* pv. *glycinea* [19]), *P. syringae* pv. *glycinea* strain 2159 and *X. campestris* pv. *glycines* strain 554 were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), which is maintained at The Harpenden Laboratory, Hatching Green, Harpenden, Herts., England. *P. syringae* pv. *glycinea* strains K1, K2, K4, K6, and K7 were supplied by B. Kennedy, Department of Plant Pathology, University of Minnesota, St. Paul, whereas strains A-29-2 and J3-17-2 were isolated by the senior author from soybean fields in Wisconsin. *Pseudomonas fluorescens* strain 10B and *X. campestris* pv. *campestris* strain 42 were obtained from the culture collection of the Department of Plant Pathology, University of Wisconsin, Madison; *C. flaccumfaciens* pv. *flaccumfaciens* from M. Schuster, Department of Horticulture, University of Nebraska, Lincoln; *B. subtilis* strain 744 and *B. licheniformis* strain 1264 from the USDA Agricultural Research Culture Collection (NRRL), Peoria; *Lactobacillus bulgaricus* strain LB1, *Streptococcus lactis* strain 19435 and *S. thermophilus* strain ST18 from G. Somkuti and *Staphylococcus aureus* strain 196E from S. Palumbo, both of the USDA Eastern Regional Research Center, Philadelphia; and *X. campestris* pv. *glycines* strain XP3 from the International Collection of Phytopathogenic Bacteria (ICPPB), University of California, Davis.

P. syringae pv. *glycinea* strains K1 and NCPPB 2159 represent physiologic race 1; K2, race 2; K4 and A-29-2, race 4; J3-17-2, race 5; K6, race 6; and K7, race 7 (3,6).

Pseudomonads were grown on King's Medium B agar (14) and other bacteria on nutrient dextrose agar (NDA) (Difco nutrient agar supplemented with 10 g/L of Difco dextrose and 5 g/L of Difco yeast extract, Difco Laboratories, Detroit, MI 48232) for 24–48 hr at room temperature (approximately 24 C). A Klett-Summerson colorimeter (Klett Manufacturing Company, New York, NY) with a 640-nm red filter was used to measure turbidity of bacterial cell suspensions.

Phytoalexins. Commercially obtained coumestrol (Eastman Kodak Co., Rochester, NY 14650) was further purified by recrystallization from ethanol-water. When the purified preparation was chromatographed on 250-μm silica gel thin-layer chromatographic (TLC) plates (Analtech, Newark, DE 19711) in hexane:EtOAc:MeOH (60:40:1) or $CHCl_3$:MeOH (96:4), a single blue-fluorescent spot was present when the plate was viewed under 350-nm light. No additional spots were evident after exposure of plates to iodine vapor.

Glyceollin was isolated by a method modified from that of Ayers et al (2). Five hundred grams of seed of the soybean cultivar Clark (obtained from R. L. Bernard, United States Regional Soybean

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Laboratory, Urbana, IL 61801) was soaked for 4 hr in distilled water. The swollen seeds were chopped into quarters, sprayed with a water suspension of *Cladosporium* sp. spores (approximately 10^7 spores per milliliter), and incubated in the dark at room temperature. After seed surfaces had turned a reddish brown (3–4 days), seed quarters were immersed in 2 L of 95% ethanol and allowed to soak for 2–3 days at 4 C. The fluid, combined with an additional 200 ml of 95% ethanol used to rinse the seeds, was filtered through two layers of cheesecloth, and the combined filtrates were centrifuged at 5,860 *g* for 20 min. The supernatant was collected and evaporated under vacuum to approximately 100 ml. This sample was extracted four times with three volumes of chloroform each time. The combined chloroform extracts were evaporated to dryness under nitrogen, and the residue was taken up in 10 ml of absolute ethanol. One-milliliter samples were spotted onto 1,000-μm silica gel preparative TLC plates (20 × 20 cm) containing fluorescent indicator (Analtech, Newark, DE 19711) along with a glyceollin standard (supplied by N. T. Keen, Department of Plant Pathology, University of California, Riverside 92521). Fluorescence-quenching areas corresponding to the glyceollin standard (when viewed under 254-nm light) were scraped from the plates, packed into glass columns, and eluted with absolute ethanol. The combined ethanol extract was evaporated to dryness under nitrogen, taken up in 2 ml of methanol, and subjected to high-performance liquid chromatography (HPLC) with a Waters Associates (Milford, MA 01757) HPLC system. Fifty-microliter samples were injected into a Model U6k injector attached to a Model M-6000A Chromatography Pump and a 10-μm Porasil column. Glyceollin was eluted with 3% isopropanol in hexane (Nanograde purity; Mallinckrodt, St. Louis, MO 63134) at a flow rate of 2 ml/min. Absorbance at 285 nm was followed with a Model 450 variable wavelength detector. Peak fractions corresponding in retention time to the glyceollin standard (8.0 min—isomer I, 8.6 min—isomer II, 9.4 min—isomer III) were collected, pooled, and evaporated to dryness under nitrogen.

Mass spectra for the purified glyceollin preparations were obtained by using a Hewlett-Packard 5992B gas chromatograph/mass spectrometer.

Bioassays. *Direct spotting.* The procedure of Wyman and Van Etten (26) was followed. Bacterial cells were scraped from the agar surface, suspended in sterile water, and turbidity was adjusted to 25 Klett units (approximately 1×10^8 colony-forming units (cfu/ml). Bacterial suspensions (0.1 ml) were added to 4.9 ml of molten water agar (WA) (50 C) (1.5% w/v Difco Bacto-Agar) contained in 16 × 150-mm glass test tubes. Tube contents were mixed and poured over a solidified base of NDA contained in 100 ×15-mm disposable polystyrene culture dishes. The test solutions (5-μl volume) were applied to the seeded WA surface after the WA solidified (approximately 30 min). Coumestrol was used at a concentration of 2 mg/ml in dimethyl sulfoxide (DMSO) (Fischer Scientific, Fair Lawn, NJ 07410) and glyceollin at 1.0, 2.0, or 7.5 mg/ml in 95% ethanol. Ethanol and DMSO were used alone as controls. Streptomycin sulfate (Boehringer Mannheim, West Germany) at 2 mg/ml in water was spotted as a positive control. Plates were incubated for 48 hr at room temperature (72 hr for the slower growing *S. thermophilus* strain ST18 and *L. bulgaricus* strain LB1).

Paper disk. Bacterial suspensions (0.1 ml), prepared as for the spot bioassays, were spread over the surface of solidified Difco nutrient agar (NA) or NDA. Dish lids were left ajar until agar surfaces were dry. Fifty-microliter samples of glyceollin (1 or 2 mg/ml in 95% ethanol), 95% ethanol, or streptomycin sulfate (1 mg/ml in water) were placed on sterilized 1.27-cm-diameter filter paper disks (Schleicher and Schuell, Inc., Keene, NH 03431). After the solvents had evaporated, the filter paper disks were placed on the surface of the test medium. Inhibitory areas were determined after 48 hr of incubation at room temperature except for the slower growing *S. thermophilus* strain ST18 and *L. bulgaricus* strain LB1 (5 days).

Agar incorporation. Preliminary experiments were run on the effect of varying ethanol or DMSO concentrations in NA media on growth of *P. syringae* pv. *glycinea* strains NCPPB 2159, A-29-2, J3-17-2, *P. syringae* pv. *phaseolicola* strain NCPPB 1134 and *P. fluorescens* strain 10B. Ethanol at 3% (v/v) strongly inhibited growth by *P. syringae* pv. *phaseolicola* strain NCPPB 1134, but had only slight or no effect on other strains. At 2% (v/v), it slightly inhibited growth of *P. syringae* pv. *phaseolicola* strain NCPPB 1134, but showed no inhibition of the four other bacterial strains. A 2% (v/v) ethanol concentration in agar was used to enhance solubility of glyceollin.

DMSO at 10% (v/v) in NA completely inhibited growth of all five bacterial strains. At 5% (v/v), it partially inhibited growth of *P. syringae* pv. *glycinea* strains NCPPB 2159 and J3-17-2 and *P. fluorescens* strain 10B. At 2% (v/v), it slightly inhibited only *P. syringae* pv. *glycinea* strain J3-17-2 and *P. fluorescens* strain 10B. A concentration of 2.5% (v/v) DMSO was used to enhance coumestrol solubility.

Sixty microliters of glyceollin at 5 or 7.5 mg/ml in 95% ethanol or 75 μl coumestrol at 2 or 4 mg/ml in DMSO were added to 3 ml of molten NA or NDA contained in 100 × 13-mm glass test tubes. Tube contents were mixed and poured into 60 × 15-mm sterile disposable polystyrene culture dishes. Ethanol (final concentration of 2% v/v) or DMSO (final concentration of 2.5% v/v) was added alone as a control.

Bacterial cells were suspended in sterile water as before, but the turbidity of the cell suspensions was adjusted to 50 Klett units (approximately 5×10^8 cfu/ml). Serial dilutions were prepared in sterile water and 0.1-ml samples from the end dilution tubes were spread over the surface of the test media. Dishes were sealed with parafilm and incubated at room temperature.

Nutrient broth. Sterilized Difco nutrient broth (NB) (4.8 ml, pH 6.5) was placed in sterilized culture vessels patterned after those described by Zalewski and Sequeira (28), and 0.1 ml of glyceollin (5 mg/ml in 95% ethanol) was added (final concentration of 100 μg glyceollin per milliliter). Control flasks contained 2% ethanol or NB alone.

Bacterial cells of *P. syringae* pv. *phaseolicola* strain NCPPB 1134 and *P. syringae* pv. *glycinea* strain NCPPB 2159 were suspended in sterile water, and turbidity was adjusted to 110 Klett units. Bacterial inoculum (0.1 ml) was added to appropriate flasks to give a starting concentration of approximately 2×10^7 cfu/ml.

All flasks were placed in a controlled-temperature water bath (26 C) and shaken (100 oscillations per minute). Bacterial growth was monitored by hourly readings of optical density at 660 nm. Each bacterial strain was tested four times.

In an attempt to determine if *P. syringae* pv. *glycinea* can degrade glyceollin, all flasks were held at 4 C after the final turbidity reading (24 hr); within 2 hr, flask contents were extracted three times with five volumes of chloroform per extraction. Chloroform extracts were combined and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of ethanol, and the solution was spotted on silica gel TLC plates (250 μm with fluorescent indicator) along with a glyceollin standard. Areas corresponding to the standard were scraped from the plates, packed into glass columns, and 9 ml of ethanol was used to elute any glyceollin present. The samples were again evaporated to dryness under a stream of nitrogen, and 2 ml of ethanol was added to the residue of each sample. The absorbance of each sample at 285 nm was determined, and glyceollin content was calculated from the molar extinction coefficient of 10,300 (2). In addition to NB, peptone-water and Pseudomonas minimal medium (7) were also amended with glyceollin and used with *P. syringae* pv. *glycinea* strain NCPPB 2159. These two additional media supported little growth.

Micro-broth dilution. Concentration series of glyceollin (400, 200, 100, 50, 25, 12.5, or 6.25 μg per well) were prepared in "Multiwell" tissue culture plates (24 wells per plate) (Becton Dickinson and Company, Oxnard, CA 93030). Plate lids were left ajar until the solvent (95% ethanol) had evaporated.

Bacterial cells were suspended in NDB (pH 6.5), and turbidity was adjusted to 25 Klett units. Ten microliters of the suspension was added to 10 ml NDB with or without 2% v/v ethanol as a solubilizing agent. One milliliter of the bacterial inoculum (containing approximately 10^5 cfu/ml) was added to each well. For each bacterial strain tested, two control wells without

glyceollin were also inoculated. Plates were shaken at 40 oscillations per minute for 24 hr at room temperature. After incubation, two 0.1-ml samples of undiluted culture and from each tube of three serial 1:100 dilutions prepared in 0.1% peptone-water from each well were plated on two culture dishes containing NDA. Dishes were incubated at 28 C for 48 hr, and the resultant bacterial colonies were counted. Bacterial strains were tested once under each assay condition.

Peptone-water. Bacterial cells were washed three times with 0.1% (w/v) Difco Bacto-peptone in water (pH 6.5). Turbidity of the suspension was adjusted to 110 Klett units, and 0.1 ml was added to 9.9 ml of peptone-water plus 2% (v/v) ethanol (final bacterial concentration of approximately 10^7 cfu/ml). Further procedures were the same as those for the micro-broth dilution bioassay except that platings of undiluted and diluted samples were made after 4 and 24 hr of incubation. The assay was repeated at least one time for each bacterial strain.

RESULTS

The identity and purity of the glyceollin preparation from Clark soybean seed challenged with *Cladosporium* sp. was confirmed by comparison with authentic glyceollin by three separate methods: cochromatographing on silica gel TLC plates (250 μm) with fluorescent indicator and using hexane:EtOAc:MeOH (60:40:1), MeOH:hexane (9:1) or $CHCl_3$:MeOH (98:2) for development; gas chromatography-mass spectrometry of the trimethylsilyl derivatives; and comparing ultraviolet absorption spectra. On silica gel TLC plates, the purified seed extract of glyceollin gave a single background fluorescence-quenching spot under 254-nm illumination and it was the only visible spot after staining with iodine vapors. Gas chromatography of the trimethylsilyl derivatives indicated a purity of at least 95% and an isomeric composition of approximately 70%-I, 20%-II, and 10%-III (15). The glyceollin sample obtained from N. Keen had an isomeric composition of approximately 10%-I, 30%-II, and 60%-III. The mixture of glyceollin isomers from seeds was used in all bioassays unless otherwise stated.

Glyceollin concentration was determined by dry weight and confirmed by absorption at 285 nm and a molar extinction coefficient of 10,300 in ethanol (2). Coumestrol concentration was determined by dry weight alone.

The seed extract of glyceollin inhibited *Cladosporium* sp. growth at 0.5 μg and above when bioassayed on silica gel TLC plates (13).

Direct spotting bioassay. The Gram-positive bacteria *B. licheniformis* strain 1264, *B. subtilis* strain 744, *S. aureus* strain 196E, *S. lactis* strain 19435, and *S. thermophilus* strain ST18 were inhibited moderately to strongly by glyceollin at 10 μg/ml (Table 1). The Gram-positive bacterium *L. bulgaricus* strain LB1 was not inhibited by 5 μg of glyceollin per milliliter and inhibited only weakly by 10 μg per milliliter. The Gram-negative saprophyte *P. fluorescens* strain 10B was not inhibited by 10 μg of glyceollin.

Of the bacteria pathogenic towards soybean, all four of the *P. syringae* pv. *glycinea* strains were insensitive to glyceollin up to 37.5 μg, but *X. campestris* pv. *glycines* strains XP 3 and NCPPB 554 were inhibited by both 5 and 10 μg of glyceollin. The heterologous plant pathogenic bacterium *X. campestris* pv. *campestris* strain 42 was also inhibited by 5 and 10 μg of glyceollin. *P. syringae* pv. *phaseolicola* strain 1134 was not affected by glyceollin. No data could be obtained for *C. flaccumfaciens* pv. *flaccumfaciens* in this bioassay because the bacterium was strongly inhibited by 95% ethanol alone.

Coumestrol, at 10 μg, completely inhibited growth of *B. licheniformis* strain 1264 and *B. subtilis* strain 744, but did not inhibit any of the other bacterial strains tested (Table 1). No data could be obtained for *S. aureus* strain 196E, *S. lactis* strain 19435, and *S. thermophilus* strain ST18 because all three were strongly inhibited by DMSO alone.

To test for possible synergistic inhibitory activity of glyceollin and coumestrol against *P. syringae* pv. *glycinea* and pv. *phaseolicola*, 10 μg of coumestrol was spotted directly over 10 μg of glyceollin. No inhibitory activity of the mixture against *P. syringae* pv. *glycinea* strains NCPPB 2159, K2, A-29-2, J3-17-2, and pv. *phaseolicola* strain NCPPB 1134 was noted.

Streptomycin sulfate, at 10 μg completely inhibited (rating of 4.0) all bacterial strains.

Paper disk bioassay. The paper disk bioassay was used for testing the inhibitory activity of glyceollin, but it could not be used for coumestrol because it is of limited applicability for testing highly water-insoluble compounds. Growth of all the Gram-positive bacteria tested, except for *L. bulgaricus* strain LB1, was inhibited near paper disks containing 100 μg of glyceollin (Table 2). *S. aureus* strain 196E and *S. thermophilus* strain ST18 were the most sensitive strains tested, being inhibited by 50 μg of glyceollin per paper disk. Glyceollin did not inhibit any of the Gram-negative bacteria tested including four strains of *P. syringae* pv. *glycinea*. In all cases, sensitivity to glyceollin even at 100 μg per disk was much less than to streptomycin sulfate at 50 μg.

Agar incorporation. Bacterial strains were plated on NA or NDA media containing 100 μg glyceollin per milliliter. Each strain was tested at least three times. Colony size and morphology of *P. fluorescens* strain 10B, *P. syringae* pv. *glycinea* strains K1, NCPPB 2159, K2, K4, A-29-2, K5, J3-17-2, K6, K7, *P. syringae* pv. *phaseolicola* strain NCPPB 1134, and *X. campestris* pv. *glycines* strains XP 3 and NCPPB 554 were not affected. Growth of *B. subtilis* strain 744, *B. licheniformis* strain 1264, and *C. flaccumfaciens* pv. *flaccumfaciens* was totally inhibited at this concentration. To distinguish between a bacteriostatic or bactericidal effect, 0.4 ml of sterile water was spread over the surface of plates showing no growth and then plated out on NDA alone. No growth occurred on these plates after 5 days incubation at room temperature, which indicated that glyceollin had had a bactericidal effect.

Growth of *P. fluorescens* strain 10B, *P. syringae pv. glycinea* strains NCPPB 2159, A-29-2, J3-17-2, and *P. syringae* pv.

TABLE 1. Antibacterial activity of glyceollin and coumestrol as determined by direct spotting onto agar medium seeded with bacteria

Organism	Strain	Glyceollin (μg) 5	10	37.5	Coumestrol (10 μg)
Bacillus licheniformis	1264	1.7[a]	3.0	···[b]	4.0
B. subtilis	744	1.5	3.5	···	4.0
Corynebacterium flaccumfaciens pv. *flaccumfaciens*		···	···	···	0
Lactobacillus bulgaricus	LB1	0	1.7	···	0
Staphylococcus aureus	196E	1.0	4.0	···	···
Streptococcus lactis	19435	1.3	4.0	···	···
S. thermophilus	ST18	1.7	3.7	···	···
Pseudomonas fluorescens	10B	0	0	···	0
P. syringae pv. *glycinea*	2159	0	0	0	0
	K2	0	0	0	0
	A-29-2	0	0	0	0
	J3-17-2	0	0	0	0
pv. *phaseolicola*	1134	0	0	0	0
Xanthomonas campestris pv. *campestris*	42	1.3	2.0	···	0
X. campestris pv. *glycines*	3	2.0	3.0	···	0
	554	1.0	2.0	···	0

[a] Rating scale used based on bacterial growth inhibition: 0 = no inhibition, 1 = trace of activity, 2 = moderate activity, 3 = strong activity, and 4 = complete inhibition of growth. Values shown are the average results of at least three separate determinations.

[b] (···) means assay not run or invalid due to inhibitory activity of ethanol or dimethyl sulfoxide alone (see text).

phaseolicola strain NCPPB 1134 was not inhibited when cells were plated onto NA containing 150 μg glyceollin per milliliter. Additionally, these strains plus *P. syringae* pv. *glycinea* strains K1, K2, K4, K6, and K7 were plated onto NA containing 100 μg/ml of glyceollin from N. T. Keen to see if glyceollin enriched in isomer III might be inhibitory. Results were negative.

Experiments testing the effects of coumestrol incorporated into agar medium at 50 or 100 μg/ml were repeated at least once. Coumestrol was not completely soluble in NA or NDA at these concentrations as determined by microscopic observation. At 50 μg/ml, coumestrol completely inhibited growth of *B. subtilis* strain 744, *B. licheniformis* strain 1264, *S. aureus* strain 196E, and *S. thermophilus* strain ST18. *P. fluorescens* strain 10B, *P. syringae* pv. *glycinea* strains NCPPB 2159, K2, A-29-2, J3-17-2, *P. syringae* pv. *phaseolicola* strain NCPPB 1134, *X. campestris* pv. *campestris* strain 42, *X. campestris* pv. *glycines* strains XP 3 and NCPPB 554, *C. flaccumfaciens* pv. *flaccumfaciens*, *L. bulgaricus* strain LB1, and *S. lactis* strain 19435 were unaffected. When selected strains that were unaffected by 50 μg/ml (*P. fluorescens* strain 10B, *P. syringae* pv. *glycinea* strains NCPPB 2159, K2, and A-29-2 plus *X. campestris* pv. *glycines* strains XP 3 and NCPPB 554) were tested against coumestrol at 100 μg/ml in NDA, only *X. campestris* pv. *glycines* strain XP 3 was affected, with total inhibition of growth. The inhibitory effect of coumestrol also appeared to be bactericidal when tested by the previous method.

Comparable results were obtained when NA or NDA media were used. In all trials, where no inhibition of bacterial growth occurred, approximately 20 to 100 bacterial colonies formed per culture dish.

TABLE 2. Antibacterial activity of glyceollin as determined by a filter paper disk bioassay

Organism	Strain	Area of inhibition (cm^2)[a] Glyceollin (μg) 50	Glyceollin (μg) 100	Streptomycin (μg) 50
Bacillus licheniformis	1264	0[b]	1.02	5.92
B. subtilis	744	0	0.87	2.62
Corynebacterium flaccumfaciens pv. *flaccumfaciens*		0	1.22	5.69
Lactobacillus bulgaricus	LB1	0	0	0.74
Staphylococcus aureus	196E	0.38	1.27	1.88
Streptococcus lactis	19435	0	0.74	1.42
S. thermophilus	ST18	0.74	2.36	3.44
Pseudomonas fluorescens	10B	0	0	2.65
P. syringae pv. *glycinea*	2159	0	0	5.51
	K2	0	0	5.51
	A-29-2	0	0	5.22
	J3-17-2	0	0	5.51
pv. *phaseolicola*	1134	0	0	5.20
Xanthomonas campestris pv. *campestris*	42	0	0	4.45
pv. *glycines*	3	0	0	7.04
	554	0	0	4.72

[a] Area of inhibition = area of inhibitory region minus area of paper disk.
[b] Values shown are the average results of at least two separate determinations.

TABLE 3. Antibacterial effect of glyceollin as determined by micro-broth dilution assay

Organism	Strain	No ethanol MIC[a]	No ethanol MBC[b]	2% Ethanol MIC	2% Ethanol MBC
Bacillus subtilis	744	<6.25[c]	50	<6.25	50
Corynebacterium flaccumfaciens pv. *flaccumfaciens*	...	<6.25	100	<6.25	100
Staphylococcus aureus	196E	<6.25	100	<6.25	100
Pseudomonas syringae pv. *glycinea*	2159	400	>400	400	>400
	A-29-2	12.5	>400	12.5	>400
Xanthomonas campestris pv. *campestris*	42	12.5	400	<6.25	400
pv. *glycines*	554	100	>400	50	400

[a] MIC = minimum inhibitory concentration, defined as lowest concentration of glyceollin which inhibited growth.
[b] MBC = minimum bactericidal concentration, defined as lowest concentration of glyceollin which caused complete killing of the bacteria.
[c] Data reported as micrograms of glyceollin per milliliter nutrient dextrose broth (pH 6.5).

Nutrient broth bioassay. For *P. syringae* pv. *phaseolicola* strain NCPPB 1134, mean lag time was 3.7 hr with no addition to NB, 4.0 hr with addition of 2% (v/v) ethanol, and 4.8 hr with addition of 100 μg glyceollin per milliliter and 2% (v/v) ethanol. Mean doubling times for the three treatments were 2.1, 2.7, and 3.3 hr, respectively. For *P. syringae* pv. *glycinea* strain NCPPB 2159, mean lag time was 4.3 hr with no addition to NB, 4.0 hr with the addition of 2% (v/v) ethanol, and 4.5 hr with the addition of 100 μg glyceollin per milliliter and 2% (v/v) ethanol. Mean doubling times for the three treatments were 2.7, 3.1, and 3.8 hr, respectively. Analysis of variance indicated no significant F values ($P = 0.05$) for treatment effects for either lag or doubling times for the *P. syringae* pv. *glycinea* and pv. *phaseolicola* strains.

In all media, approximately 80 to 100% of the control glyceollin levels were recovered from flasks inoculated with *P. syringae* pv. *glycinea* strain NCPPB 2159, indicating that no significant degradation had occurred.

Micro-broth dilution bioassay. *B. subtilis* strain 744 was the most sensitive bacterium tested, with a minimum inhibitory concentration (MIC) of <6.25 μg glyceollin per milliliter and a minimum bactericidal concentration (MBC) of 50 μg glyceollin per milliliter both in the presence and absence of ethanol (Table 3). *C. flaccumfaciens* pv. *flaccumfaciens* and *S. aureus* strain 196E were only slightly less sensitive to the effects of glyceollin with MIC's of <6.25 μg glyceollin per milliliter and MBC's of 100 μg glyceollin per milliliter. *X. campestris* pv. *campestris* strain 42 (MIC = <6.25 μg glyceollin per milliliter, MBC = 400 μg glyceollin per milliliter in the presence of ethanol), *X. campestris* pv. *glycines* strain 554 (MIC = 50 μg glyceollin per milliliter, MBC = 400 μg glyceollin per milliliter in the presence of ethanol) and *P. syringae* pv. *glycinea* strain A-29-2 (MIC = 12.5 μg glyceollin per milliliter, MBC = >400 μg glyceollin per milliliter) were intermediate in sensitivity toward glyceollin. *P. syringae* pv. *glycinea* strain NCPPB 2159 was the least affected strain tested (MIC = 400 μg glyceollin per milliliter, MBC = >400 μg glyceollin per milliliter both in the presence and absence of ethanol).

In the presence of 400 μg glyceollin per milliliter approximately 18 and 30% of control growth values were obtained for *P. syringae* pv. *glycinea* strains A-29-2 and NCPPB 2159, respectively. Strain NCPPB 2159 was also tested against 800 μg/ml and 1.6 mg/ml of glyceollin with similar results. Glyceollin was not completely soluble at these two concentrations even when 2% (v/v) ethanol was added.

Peptone-water bioassay. The findings of a single experiment are shown in Table 4; other repetitions gave similar results. *B. subtilis* strain 744 was the most sensitive strain tested. No cells of this strain survived 4 hr incubation with 100 or 200 μg/ml of glyceollin. *P. syringae* pv. *phaseolicola* strain NCPPB 1134 was intermediate in sensitivity and *P. syringae* pv. *glycinea* strain NCPPB 2159 was unaffected by all glyceollin levels.

DISCUSSION

The soybean isoflavonoids coumestrol and glyceollin were found to be inhibitory to several strains of both Gram-positive and Gram-negative bacteria. Gram-positive bacteria generally were more

sensitive to the effects of coumestrol than were Gram-negative bacteria. In the agar incorporation bioassay, coumestrol was bactericidal towards strains of *B. subtilis*, *B. licheniformis*, *S. aureus*, and *S. thermophilus*. *X. campestris* pv. *glycines* was the only Gram-negative bacterium inhibited, and only at the higher of the two coumestrol concentrations tested.

Coumestrol has been reported to be inhibitory towards the plant pathogenic bacteria *P. syringae* pvs. *glycinea* (8), *mors-prunorum* (18) and *phaseolicola* (8,16), and to a lesser degree the saprophyte *P. fluorescens* (8,16). Wyman and VanEtten (26) found no inhibitory activity of coumestrol against a broad range of bacteria including strains of pv. *glycinea*, pv. *phaseolicola*, and *P. fluorescens*, agreeing with our findings.

Glyceollin inhibited growth of all bacteria tested except *P. fluorescens* strain 10B. However, this bacterium was not tested in bioassays using liquid media. The inhibition of several bacterial strains was due to a bactericidal effect.

Glyceollin inhibited growth of Gram-positive bacteria more than Gram-negative bacteria as demonstrated by results of the micro-broth dilution bioassay. The Gram-positive bacteria *B. subtilis* strain 744, *C. flaccumfaciens* pv. *flaccumfaciens* and *S. aureus* strain 196E had lower minimum bactericidal concentrations of glyceollin than the Gram-negative *P. syringae* pv. *glycinea* strains A-29-2 and NCPPB 2159, *X. campestris* pv. *campestris* strain 42, and *X. campestris* pv. *glycines* strain NCPPB 554. Gnanamanickam and Smith (9) recently reported highly selective antibacterial activity of the bean (*Phaseolus vulgaris* L.) isoflavonoids kievitone, phaseollin, phaseollinisoflavan, and phaseollidin against Gram-positive bacteria. These findings plus the results reported here indicate that the target site of the inhibitory action of isoflavonoid phytoalexins may be more accessible or more sensitive in Gram-positive than in Gram-negative bacteria. Albersheim and Valent (1) reported, however, that glyceollin was equally active against Gram-negative *P. syringae* pv. *glycinea* and Gram-positive *B. subtilis*.

Several bacteria were shown to be more sensitive to the inhibitory effects of glyceollin than the soybean pathogen *Phytophthora megasperma* var. *sojae*. For example, Yoshikawa et al (27) reported that a minimum concentration of 300 μg glyceollin per milliliter was needed to completely arrest mycelial growth of *P. megasperma* var. *sojae*, whereas in our micro-broth dilution bioassay, *B. subtilis* strain 744 was completely inhibited by 50 μg of glyceollin per milliliter.

Results of the three bioassays for inhibitory effects of glyceollin in agar media (direct spotting onto agar media seeded with bacteria, paper disk, and agar incorporation) showed that the direct spotting bioassay of Wyman and Van Etten (26) was most effective, although its use may be limited due to toxicity of solubilizing agents. Several bacteria that were inhibited by glyceollin in the spot bioassay were either not inhibited in the paper disk and agar incorporation bioassays or inhibited only by higher concentrations of glyceollin. All three bioassays that utilized agar media did not show any inhibitory effect of glyceollin on *P. syringae* pv. *glycinea*, however.

In bioassays with liquid media, an inhibitory effect of glyceollin on pv. *glycinea* was demonstrated. *P. syringae* pv. *glycinea* strains A-29-2 and NCPPB 2159 differed markedly in their sensitivity to glyceollin with strain NCPPB 2159 being the more insensitive. Glyceollin inhibited growth of *P. syringae* pv. *glycinea* strain NCPPB 2159 only at 400 μg/ml. This concentration is much higher than that reported to inhibit *P. syringae* pv. *glycinea* by Keen and Kennedy (12) and Albersheim and Valent (1). Keen and Kennedy (12) found that glyceollin incorporated into nutrient agar medium at 100 μg/ml was bacteriostatic towards *P. syringae* pv. *glycinea* races 1, 2, and 5 and *X. campestris* pv. *glycines*. Coumestrol at 50 μg/ml was also inhibitory, but to a lesser degree. Albersheim and Valent (1) reported that glyceollin at approximately 34 μg/ml inhibited growth of *P. syringae* pv. *glycinea* in liquid media by 50%. However, Wyman and Van Etten (26) reported that *P. syringae* pv. *glycinea* was insensitive to the structurally similar bean isoflavonoid phytoalexins.

Keen and Kennedy (12) used bioassays and races of *P. syringae* pv. *glycinea* similar to those used in this study, but reported markedly different results. Even though members of the same races of *P. syringae* pv. *glycinea* were employed, the particular strains may have differed. Strain specificity of inhibition by isoflavonoid phytoalexins has been reported (8,26) and was demonstrated in this study for *P. syringae* pv. *glycinea* and glyceollin. Another possibility for the differing results between the present study and that of Keen and Kennedy (12) is that the isomeric composition of the glyceollin mixtures used in the studies differed. We tested glyceollin enriched both in isomer I and isomer III, but the isomeric composition of glyceollin used in the other study was not stated. It is probable, however, that owing to their close structural similarity (15), glyceollin isomer II would be as toxic as isomer I. Kaplan et al (10) found that glyceollin isomers I and II were equally inhibitory towards motility of the nematode *Meloidogyne incognita* and that isomer III was less active.

TABLE 4. Survival of bacteria in peptone-water amended with glyceollin

Glyceollin (μg/ml)	Percentage of control survival after:					
	Bacillus subtilis strain 744		*P. syringae* pv. *phaseolicola* strain 1134		*P. syringae* pv. *glycinea* strain 2159	
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
6.25	100	80	97	100	100	100
12.5	61	38	94	100	100	100
25	48	17	88	100	100	100
50	15	<1	88	18	100	100
100	0	0	18	3	100	100
200	0	0	<1	<1	100	100

Even though in vitro bioassays do not necessarily reflect in vivo phenomena, these findings suggest that the role of coumestrol and glyceollin in race-specific resistance of soybean to *P. syringae* pv. *glycinea* in vivo (12) requires further study. By homogenizing leaf tissue inoculated with an incompatible strain of *P. syringae* pv. *glycinea*, Keen and Kennedy (12) reported maximum glyceollin levels of approximately 1,200 μg/g fresh weight. However, after using a facilitated diffusion technique, which may more accurately reflect actual levels of glyceollin in intercellular leaf spaces, Keen (11) reported that glyceollin levels reached only approximately 200–400 μg/g fresh weight for several incompatible *P. syringae* pv. *glycinea*-soybean cultivar interactions. In the present study, in vitro growth of *P. syringae* pv. *glycinea* strain NCPPB 2159 was unaffected below 400 μg/ml of glyceollin. It is critical that the levels of glyceollin to which the bacteria are exposed in vivo be determined.

In concurrence with the findings of Keen and Kennedy (12), we found that glyceollin and coumestrol inhibited the growth of *X. campestris* pv. *glycines*. Glyceollin and coumestrol may play a role in the resistance of soybean to this bacterial pathogen. The greatly increased levels of glyceollin and coumestrol upon invasion of soybean leaves by *P. syringae* pv. *glycinea* (12) may also be important in inhibiting growth of secondary bacterial invaders.

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