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Host-Pathogen Interactions[1]

XVIII. ISOLATION AND BIOLOGICAL ACTIVITY OF GLYCINOL, A PTEROCARPAN PHYTOALEXIN SYNTHESIZED BY SOYBEANS

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ABSTRACT

A previously unrecognized phytoalexin has been isolated from soybean cotyledons that had been infected with bacteria or exposed to ultraviolet light. The phytoalexin has been purified to homogeneity by silica gel flash chromatography and high pressure liquid chromatography. It has been structurally characterized by its ultraviolet, circular dichroism and nuclear magnetic resonance spectra, polarimetry, and its mass spectrometric fragmentation pattern. The phytoalexin, (6a*S*,11a*S*)-3,6a,9-trihydroxypterocarpan, is a compound that had previously been detected in $CuCl_2$-treated soybeans and is structurally related to the previously identified soybean phytoalexins glycerollins I to IV. It is proposed that the trivial name glycinol be used for this phytoalexin. Glycinol is a broad spectrum antibiotic capable of prolonging the lag phase of growth of all six bacteria examined, namely *Erwinia carotovora*, *Pseudomonas glycinea* (races 1 and 3), *Escherichia coli*, *Xanthomonas phaseoli*, and *Bacillus subtilis*. Glycinol also inhibits the growth of the fungi *Phytophthora megasperma* f. sp. *glycinea* (race 1), *Saccharomyces cerevisiae*, and *Cladosporium cucumerinum*. Glycinol is a static agent against the six bacterial species listed above and against *S. cerevisiae*, and appears to be static against the other fungi examined. As with other phytoalexins, there is no correlation between the pathogenicity of a microorganism and its sensitivity to glycinol.

Phytoalexins are low mol wt antimicrobial compounds produced by plants in response to challenge by microorganisms. These compounds accumulate at the site of infection and are involved in the plant's defense response to potential pathogens (3, 8, 14).

The accumulation of the pterocarpan phytoalexins, glyceollin[4] (Fig. 1), by soybean (*Glycine max*) in response to fungal attack has been well documented (12). *In vitro* studies have shown glyceollin to be inhibitory to the growth of both bacteria and fungi (1, 11, 12).

The major antibacterial compound accumulated when soybean cotyledons are challenged by the bacterium *Erwinia carotovora* is not glyceollin. We have purified the major antibacterial compound and have identified it as (6a*S*,11a*S*)-3,6a,9-trihydroxypterocarpan, for which we propose the trivial name glycinol. This pterocarpan has been previously observed as a quantitatively minor constituent in $CuCl_2$-treated soybean cotyledons, although no antifungal activity was recognized to be associated with it (18). The present study describes an improved isolation procedure for the pterocarpan leading to mg quantities of the crystalline compound. We present also the results of our studies of the effect of glycinol on growth of a variety of microorganisms.

MATERIALS AND METHODS

Plant Material. Certified quality soybean seed (*Glycine max* [L.] Merr. cv. Wayne) was obtained from Wilkens Seed Co. Pontiac, IL. The seed was stored in closed containers at 4 C and hand sorted for soundness before planting. The sorted seeds were planted at a density of about 75 g seed/tray (40 × 30 × 9 cm) on a 2-cm layer of potting soil (Bacto Potting Medium, Michigan Peat Co., Houston, TX) overlaying a water-soaked 4-cm layer of vermiculite (W. R. Grace and Co., Cambridge, MA). The seed was then overlayed with a 2 cm layer of vermiculite, and watered with 400 ml deionized H_2O/tray. The seedlings were grown in a Percival model PGW-108 growth chamber with a regime of 14 h of illumination (4,300 lux) at 24 C and 10 h of darkness at 18 C. The RH was maintained at about 75%. Each tray was watered with 200 ml deionized H_2O the 2 days following planting, and 400 ml thereafter.

Microorganisms. *E. carotovora* (Jones) Holl. (ATCC 495) and *Xanthomonas phaseoli* subsp. *sojensis* (ATCC 17915) were obtained from the Americian Type Culture Collection and were maintained on TSB (Baltimore Biological Labs) agar slants and plates. Wild type *Bacillus subtilis* (Ehren.) Cohn F-11 was obtained from A. Kaji, Department of Agricultural Chemistry, Kagawa University, Kagawa, Japan, and maintained on TSB. *Escherichia coli* (Migula) Cast. and Chalm. K10, obtained from R. R. Fall, University of Colorado, was maintained on *Erwinia* minimal media (EMM), which contains 10 mg nicotinic acid (Sigma), 200 mg $MgSO_4 \cdot 7H_2O$, 28 mg $FeSO_4 \cdot 7H_2O$, 3 mg $ZnSO_4 \cdot 7H_2O$, 1 g NH_4Cl, 4 g KH_2PO_4, 4 g K_2HPO_4, and 10 g glucose/liter H_2O. EMM was employed in all studies with *E. coli* and *E. carotovora*. Studies with *X. phaseoli* and *B. subtilis* were performed in TSB. *Pseudomonas glycinea* Coerper race 1 (incompatible on *Glycine max* cv. Harasoy-63) and race 3 (compatible on *G. max* c. Harasoy-63) were supplied by B. J. Costanho of the Monsanto Agricultural Products Co. *P. glycinea* cultures were maintained in a medium containing 20 g Protease Peptone No. 3 (Difco), 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, and 1 g glycerol/liter H_2O. Davis minimal medium (16) was employed for studies of the inhibition of *P. glycinea* by glycinol. All bacterial cultures were incubated at 22 C on a New Brunswick Scientific model G2 Gyrotory shaker at 250 rpm.

Phytophthora megasperma Drechs. f. sp. *glycinea* Kuan and Erwin (13) (formerly *P. megasperma* Drechs. var. *sojae* A. A.

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[4] Abbreviations: glyceollin, glyceollins I to III (Fig. 1) migrate as a single spot on TLC and are collectively referred to as glyceollin throughout this paper; TSB, trypticase soy broth; EMM, *Erwinia*-defined medium; HPLC, high pressure liquid chromatography; CFU, colony-forming unit.

Hildb.) race 1 was obtained from N. T. Keen, University of California, Riverside. Cultures were maintained and glycinol inhibition assays performed on V8 agar plates (5) at 25 C. *Cladosporium cucumerinum* Ellis and Arthur supplied by B. J. Costanho was maintained on strained green bean agar plates (400 ml of the juice surrounding canned green beans and 15 g of agar/liter of H_2O). Glycinol inhibition assays were performed in the same medium at 25 C. *Saccharomyces cerevisiae* Hansen was maintained and liquid culture inhibition assays performed at 22 C in a medium composed of 3 g Casamino acids (Difco), 5 g yeast extract (Difco), and 20 g sucrose/liter of H_2O (20).

Chemicals. A mixture of glyceollin isomers I to III (Fig. 1) was obtained as described previously (2). Synthetic daidzein and genistein were the gift of K. Kolonko, University of Colorado. The β-glucan elicitor from the mycelial walls of *P. megasperma* f. sp. *glycinea* (22) was the gift of B. S. Valent, University of Colorado. All solvents used were of reagent grade, or better.

General Methods. Ultraviolet irradiation of soybean cotyledons was performed by exposure to a General Electric model G30T8 germicidal lamp at a distance of 70 cm.

HPLC was performed using a Waters Associates model 6000A solvent delivery system. Sample injection was accomplished using a Valco model CV-6-UHPa-N60 7,000 p.s.i.g. injection value equipped with a 500-μl injection loop. Column eluent was monitored at 287 nm by a Waters Associates model 450 flow-through variable wavelength detector. Preparative scale HPLC purifications employed a Whatman Partisil-10 M9 (10 μm silica; 9.4 mm i.d. × 500 mm) column. Analytical HPLC was performed on a Waters Associates μPorasil (10 μm silica; 4.6 mm i.d. × 250 mm) column. Peak areas were determined by a Spectra-Physics Minigrator linked directly to the output of the variable wavelength detector. Guard columns (2 mm i.d. × 70 mm), used for all HPLC, were dry-packed with a silica pellicular packing material (Whatman HC-Pellosil).

UV absorption spectra of glycinol in absolute ethanol were obtained on a Cary model 17 recording spectrophotometer at a scan rate of 0.2 nm/s. The ^{1}H-NMR spectrum of glycinol was obtained in acetone-d_6 (Stohler Isotope Chemicals) using tetramethylsilane as reference on a Nicolet 360 MHz Fourier Transform NMR. Low resolution electron impact mass spectral analyses were obtained at an ionization voltage of 70 ev on a Hewlett-Packard 5980 A mass spectrometer (probe temperature ≃180 C) coupled to a Hewlett-Packard 5934 A data system. The high resolution mass spectrum of glycinol was obtained on a Varian MAT 311 A mass spectrometer (probe temperature 160 C) linked to a Varian SS-100 data system. The circular dichroism spectrum of glycinol was obtained on a Jasco J-41C spectropolarimeter with a 1-cm path length at a scan rate of 10 nm/min (Concentration = 1.1×10^{-4} M). The optical rotation of glycinol was determined at 578 nm on a Perkin-Elmer model 141M polarimeter (Concentration = 0.22 g/100 ml).

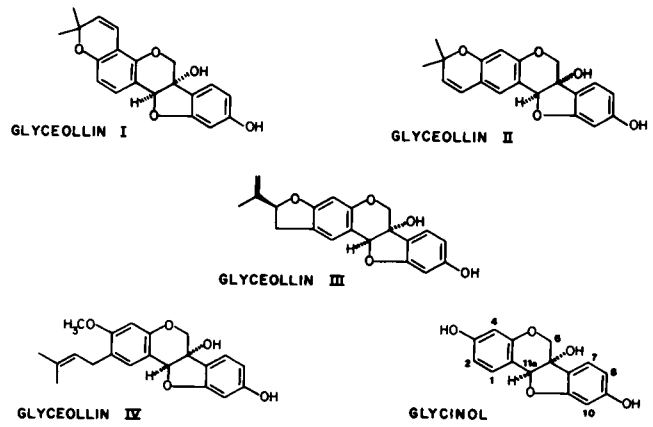

FIG. 1. Pterocarpan phytoalexins of soybean.

Biological Assays. The cotyledon assay for phytoalexin production was performed as described (2, 9) using cotyledons from 7- or 8-day-old soybean seedlings. A 90-μl sample was applied to each cotyledon and the cotyledons were incubated 20 h in the dark at 26 C. The wound-droplet solutions from the cotyledon assay were analyzed for phytoalexins either by TLC or by analytical HPLC. Wound-droplet solutions from 20 cotyledons were extracted three times with an equal volume of ethyl acetate. The ethyl acetate soluble material was evaporated to dryness under filtered air. The qualitative or quantitative content of glycinol in the residue was determined by TLC and HPLC, respectively. For TLC, the dried residue was redissolved in 200 μl ethyl acetate and aliquots of this solution (usually 5–20 μl) were applied to precoated analytical silica gel TLC plates (20 × 20 cm, Silica Gel 60 F-254, E. Merck). The plates were developed in toluene:chloroform:acetone (40:25:50, v/v/v) or hexane:ethyl acetate (30:70, v/v). Compounds were visualized under both short (λ_{max} = 254 nm) and long (λ_{max} = 366 nm) wavelength UV light.

Antibacterial compounds present on the TLC plate were identified using a modified plate bioassay procedure of Lund and Lyon (17). For this assay, an aliquot (0.5 ml) of a 24-h culture of *E. carotovora* grown on TSB was inoculated into 20 ml fresh TSB medium. This suspension was sprayed onto the TLC plate (20 × 20 cm) and the plate was incubated in a box lined with wet paper towels for 24 h at 26 C. The plate was then dried under a stream of warm air until the silica gel layer was no longer translucent (but not completely dry). Ten ml aesculin broth (2.0 mg aesculin [Pfaltz and Bauer], 1.0 mg ammonium citrate, 1.0 mg ferric citrate, 5 mg yeast extract [Difco], and distilled H_2O to 10 ml) was sprayed onto the plate. The plate was placed again in the humid incubation box for 4 to 8 h at 26 C. Areas where the bacteria were inhibited appeared under short wavelength UV light as white fluorescent spots on a brown, nonfluorescent background.

Quantitative determination of glycinol present in the ethyl acetate extract of the wound droplet solution was accomplished by redissolving the dried extract in 50 μl ethyl acetate and resolution of glycinol by HPLC. The glycinol was evaporated to dryness, redissolved in absolute ethanol, and quantitated by its *A* at 287 nm (ϵ_{287} = 5800).

Inhibition of Microbial Growth in Liquid Culture. Liquid culture inhibition assays were carried out in 50-ml culture flasks having a Bausch and Lomb 12 × 100 mm test tube attached as a sidearm. The microbial suspension was tipped into the sidearm at various intervals and turbidity read on a Bausch and Lomb Spectronic 20 at 580 nm using uninoculated media as a blank.

The antibacterial activity of glycinol was measured in either of two ways. In one protocol, glycinol was added, in a maximum of 25 μl absolute ethanol, to 4.5 ml medium. Exponentially growing bacteria or yeast (0.5 ml a liquid culture having an $A_{580\ nm}$ between 0.1 and 0.2) were added to the glycinol-containing medium. The flasks were incubated as described and the turbidity ($A_{580\ nm}$) measured at various times. A second protocol called for the addition of 0.5 ml exponentially growing bacterial or yeast culture ($A_{580\ nm}$ between 0.1 and 0.2) to 4.5 ml fresh medium with incubation of this culture as above. When the cells were observed to be in exponential growth for about 1 h, glycinol was added in a maximum of 25 μl absolute ethanol and the turbidity measurements continued. Controls, in which 25 μl absolute ethanol were added to the culture, showed no discernible effect of the ethanol on bacterial or fungal growth as assayed either by turbidity or viable counts.

Inhibition of Fungal Growth on Solid Media. The effect of glycinol on the growth of filamentous fungi was determined on agar. Glycinol at various concentrations was evenly spread over the agar surface and allowed to diffuse for about 36 h through the

agar medium (8.0 ml) in 60 × 15 mm Petri plates. A 3-mm plug of fungal mycelia was taken from the leading edge of a culture grown on the same agar medium minus glycinol and was placed in the center of either a glycinol-containing plate or a control plate. Increase in colony diameter was recorded for a maximum of 4 days.

RESULTS AND DISCUSSION

Determination of Most Effective Way to Stimulate Glycinol Accumulation in Soybean Cotyledons. The effectiveness of several methods to elicit glycinol accumulation in soybean cotyledons was compared. The treatments compared were live *E. carotovora* (≃10^9 CFU/ml), the β-glucan mycelial wall elicitor obtained from *P. megasperma* f. sp. *glycinea* (10 μg/ml), 3 mM $CuCl_2$, and 5, 10, 15, 20, 30, or 60 min exposure to UV light. Each method of elicitation results in glycinol accumulation. The most effective method tested was UV-irradiation for 30 min, which results in the accumulation of about 150 μg glycinol/g fresh weight of soybean cotyledons. Exposure of soybean cotyledons to 3 mM $CuCl_2$, the β-glucan elicitor, and live *E. carotovora* results in the accumulation of 10, 34, and 60 μg glycinol/g fresh weight, respectively. Based on these results, the procedure described in the next section has been developed for the preparative scale purification of glycinol from UV-irradiated cotyledons.

Purification of Glycinol. Cotyledons (200 g) were detached from 7- or 8-day-old soybean seedlings. A section of tissue, approximately 1 to 2 mm thick, was cut from the convex bottom surface of each cotyledon. The cotyledons were arranged with the cut surface facing up in uncovered 100 × 15 mm plastic Petri plates containing moist filter paper discs (10 cotyledons/plate) and irradiated with UV light for 30 min. Sterile, distilled H_2O (90 μl) was then placed on the cut surface of each cotyledon, the Petri plates were covered, and the cotyledons incubated for about 20 h at 26 C. The cotyledons, with the wound-droplets, were transferred from the Petri plates to an Erlenmyer flask containing 3 liters 40% ethanol. The flask was then agitated on a New Brunswick model G-25 Gyrotory shaker (≃200 rpm) for about 2 h at room temperature. Solid material was removed by successive filtrations through (a) a medium sintered glass funnel, (b) a Whatman GF/C glass fiber filter, and (c) a 0.5 μm Fluoropore (Millipore) filter. The solution obtained was concentrated to 500 ml under reduced pressure at 30 C and extracted four times with a total of 4 liters ethyl acetate. The ethyl acetate extracts were pooled, filtered through phase-separation filter paper (Whatman 1PS), and evaporated to approximately 5 ml under reduced pressure at 30 C. A precipitate which formed during this step was removed by filtration through an 0.5 μm Fluoropore filter.

Flash chromatography in hexane:ethyl acetate (30:70, v/v) of the filtered ethyl acetate solution was performed as described (21) with the exception that N_2 rather than air was used as the driving gas. Silica Gel 60 (230–400 mesh, E. Merck) was employed as the adsorbant. Fractions (12–13 ml) were collected and assayed for *A* at 287 nm. Two partially resolved peaks of 287 nm absorbing material were obtained (Fig. 2). Peak A was shown to contain predominantly glyceollin and peak B was shown to contain predominantly glycinol by direct probe MS. The fractions containing glycinol were pooled and evaporated to ≃0.5 ml under reduced pressure at 30 C.

Preparative scale HPLC (Fig. 3) of the concentrated peak B fractions was performed in a solvent system of hexane:ethyl acetate (80:20, v/v). The components were identified by comparison of retention times and mass spectrometric fragmentation patterns with those of reference compounds. The glycinol-containing peak was identified by its mass spectrum and its antibacterial activity in the *E. carotovora*/aesculin TLC plate bioassay. The glycinol solution obtained was concentrated under reduced pressure at 30 C. Glycinol was crystallized from chloroform:methanol at room temperature. Thirty mg glycinol crystals (m.p. 123–124 C) was obtained from the wound-droplets of 200 g soybean cotyledons.

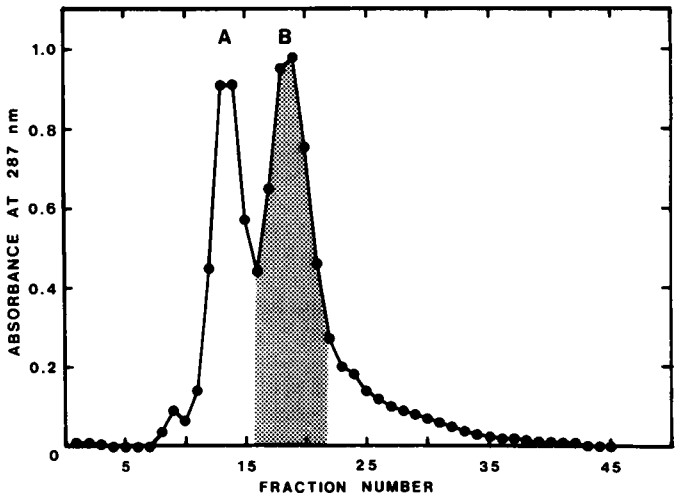

FIG. 2. Flash chromatography of the crude ethyl acetate extract of UV-irradiated soybean cotyledons. The flash chromatography was performed at ambient temperature (21). A glass column (7.0 cm i.d. × 45 cm) containing a ≃17 cm bed height of Silica Gel 60 (230–400 mesh) was overlayered with 0.5 cm sand. The extract (5 ml) was driven into the top of the silica bed under nitrogen pressure. The column was then filled with hexane:ethyl acetate (30:70, v/v) and the solvent forced through the column at a rate of ≃140 ml/min. Fractions (12–13 ml) were collected and a 1:10 dilution of each fraction was assayed as described in the text. The shaded region indicates the fractions which were pooled for further purification of glycinol.

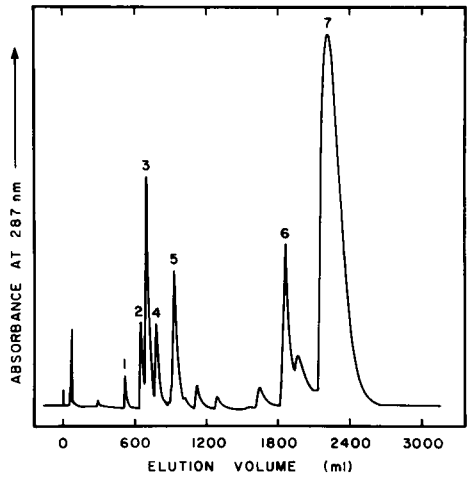

FIG. 3. HPLC of flash chromatography-purified glycinol. The HPLC on a Whatman Partisil-10 M9 column (10 μm silica; 9.4 mm i.d. × 500 mm) was performed at ambient temperature in hexane:ethyl acetate (80:20, v/v) at a flow rate of 4.0 ml/min (≃900 p.s.i.g.). The UV-absorbing peaks were identified as described in the text: 1 to 4, the glyceollin isomers (the individual isomers were not identified); 5, genistein; 6, daidzein; 7, glycinol.

Chemical Characterization of Purified Glycinol. The UV absorption spectrum of glycinol showed maxima at 282 and 287 nm with molar extinction coefficients of 5300 and 5800, respectively. The NMR spectrum of glycinol was identical with that obtained by Lyne and Mulheirn (18) for 3,6a,9-trihydroxypterocarpan. The elemental composition of glycinol was $C_{15}H_{12}O_5$ as determined by high resolution mass spectrometry of the molecular ion (found: 272.0686; calculated for $C_{15}H_{12}O_5$: 272.0685). The base peak in the spectrum of glycinol was the molecular ion, and prominent frag-

ment ions appeared at *m/z* 257 (55%), 255 (11%), 254 (40%), 253 (84%), 244 (30%), 243 (10%), 227 (20%), 213 (18%), 137 (14%). Based on the above data glycinol was identified as 3,6a,9-trihydroxypterocarpan. Polarimetric analysis showed glycinol to be levorotatory ($[\alpha]_D = -220°$). The circular dichroism spectrum of glycinol showed a positive maximum ($[\theta]_{287} = +18{,}200$). Glycinol is therefore assigned the 6a*S*,11a*S* configuration, (Fig. 1), based on comparison with other (−)-pterocarpans showing maxima in their optical rotary dispersion or circular dichroism spectra in this region (10, 23).

The R_F of purified glycinol on silica gel TLC plates was found to be 0.39 in a solvent system of toluene:chloroform:acetone (40:25:50, v/v/v) (R_F of glyceollin = 0.49) and 0.38 in a solvent system of hexane:ethyl acetate (30:70, v/v) (R_F of glyceollin = 0.46).

Biological Activity of Glycinol. The ability of glycinol to inhibit several bacteria and fungi either in solution or on solid medium was compared. Fungal radial growth inhibition assays showed that glycinol was effective in inhibiting the growth of both *P. megasperma* f. sp. *glycinea* and *C. cucumerinum* on solid media (Fig. 4). Glycinol inhibited the growth of *P. megasperma* f. sp. *glycinea* at approximately the same level of efficacy as reported previously for glyceollin (compare Figs. 4A and [12]). In the radial growth assay employed in this study, glycinol also inhibited the growth of *C. cucumerinum*. Inhibition was not observed in an earlier study of the effect of glycinol on this fungus by Lyne and Mulheirn (18) who used a TLC-plate bioassay of antimicrobial activity. In that assay, large numbers of *C. cucumerinum* spores were sprayed onto the plate and the plate incubated for 96 h before being examined for fungal growth inhibition. An explanation for these differing results may be inferred by closer examination of the data presented in Figure 4B. Glycinol inhibited the radial growth of *C. cucumerinum* for about 72 h following inoculation of the agar medium. However, 96 h after inoculation there was little difference in the diameter of *C. cucumerinum* colonies whether they were growing in the absence or the presence of glycinol, even at glycinol concentrations as high as 200 μg/ml. The previous inability to demonstrate the antifungal activity of glycinol may be due to the heavy *C. cucumerinum* inoculation and the lengthy incubation period of the TLC-plates prior to examination for regions of diminished fungal growth.

Addition of glycinol to exponentially growing bacterial cultures results in an abrupt cessation of the increase in turbidity (Fig. 7). The duration of the glycinol-induced growth inhibition is proportional to the concentration of glycinol in the medium (Fig. 5, Table I). Glycinol inhibition of the growth of *S. cerevisiae*, the only fungus of those studied capable of being examined in the liquid culture inhibition assay (Fig. 5B), showed the same pattern of inhibition as seen for bacteria. At the end of the glycinol-induced inhibition period, microbial growth resumes at the same rate as in the untreated culture (Fig. 5).

There is no correlation between the pathogenicity of a microorganism on soybean and its sensitivity to glycinol. The soybean pathogens *P. glycinea*, *X. phaseoli*, and *P. megasperma* f. sp. *glycinea* are no less sensitive to glycinol than are the nonpathogens *E. carotovora*, *E. coli*, *B. subtilis*, *S. cerevisiae*, and *C. cucumerinum*. These data indicate that sensitivity to glycinol is not the sole determinant of pathogenicity of a microbe on soybeans.

Experiments were performed to determine whether glycinol is a toxic or static agent against bacteria and fungi. Aliquots of *E. carotovora* were removed at various times during their glycinol-induced extended lag phase and were diluted and plated on trypticase soy agar. The plates were incubated for 24 to 36 h at 25 C and the colonies counted. CFUs remained constant over the entire glycinol-extended lag phase for *E. carotovora*. All of the other microbes listed in Table I examined showed the same static effect of glycinol on their growth..

The static effect of other pterocarpan phytoalexins on bacterial and fungal growth has been reported. Glyceollin (Fig. 1) has been shown to be bacteriostatic (11, 15), while pisatin, a pterocarpan phytoalexin from peas, appears to be fungistatic towards germination of *Monilinia fructicola* (4).

The limited duration of glycinol inhibition of microbial growth

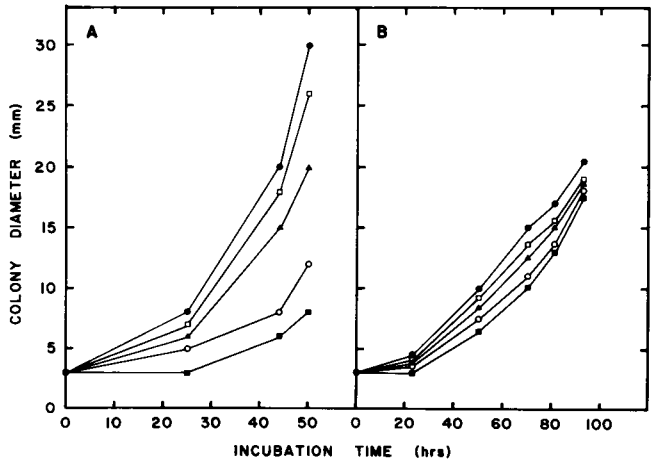

FIG. 4. Effect of various concentrations of glycinol in solid media on the growth of (A) *P. megasperma* f. sp. *glycinea* (race 1) and of (B) *C. cucumerinum*. The values for colony diameter are the average of three measurements, in different directions, of the colony diameter on each plate. The symbols represent 0 (●), 25 (□), 50 (▲), 100 (○), and 200 (■) μg/ml glycinol. The experiments were performed as described in the text.

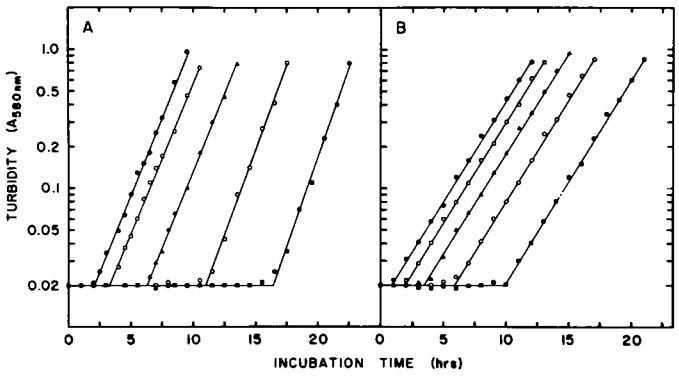

FIG. 5. Effect of various glycinol concentrations on the growth of (A) *E. carotovora* and (B) *S. cerevisiae* in liquid culture. The symbols represent 0 (●), 25 (□), 50 (▲), 100 (○), and 200 (■) μg/ml glycinol. The experiments were performed as described in the text.

Table I. *Ability of Glycinol to Inhibit Growth of Various Microorganisms*

The data are a measure of the degree of inhibition which was determined by dividing the time required for a glycinol-containing culture to reach an $A_{580\,nm}$ of 0.5 by the time required for an uninhibited culture to reach the same turbidity. Times for cultures with no glycinol added to reach an $A_{580\,nm}$ of 0.5 were 5.7, 12.9, 12.3, 9.2, 6.9, 11.7, and 9.6 h for microorganisms 1 through 7, respectively.

Microorganism	Glycinol Concentration μg/ml			
	25	50	100	200
1. *E. carotovora*	1.2	1.5	2.0	2.6
2. *P. glycinea* race 1	1.2	1.3	1.7	2.2
3. *P. glycinea* race 3	1.2	1.4	1.7	2.2
4. *E. coli* K-10	1.1	1.3	1.6	1.7
5. *B. subtilis* F-11	1.1	1.2	1.4	1.9
6. *X. phaseoli*	1.1	1.2	1.4	1.8
7. *S. cerevisiae*	1.1	1.3	1.5	1.9

in liquid culture (Fig. 5 and Table I) could be explained by either chemical decomposition of glycinol in the medium or by microbial detoxification. Microbial growth could then resume when the concentration of glycinol in the medium decreases below some threshold value. Experiments were performed to determine whether glycinol is chemically unstable in the growth media or is detoxified. In one experiment, a sidearm culture flask containing 4.5 ml EMM containing 100 mg/ml glycinol was incubated with shaking (250 rpm) at 22 C for 10 h. A fresh otherwise identical flask was prepared at the end of the 10-h preincubation period. Both flasks were then inoculated with *E. carotovora* ($\simeq 10^5$ CFU) as described under "Materials and Methods." Turbidity measurements ($A_{580\ nm}$) of subsequent growth at 22 C were identical in the two flasks. Therefore, preincubation of glycinol in the medium in the absence of bacteria does not affect the phytoalexin's ability to inhibit bacterial growth during the time span of our experiments. This result suggests glycinol is chemically stable in the culture medium.

A second experiment was set up to determine whether, and at what rate glycinol was destroyed in EMM in the presence and absence of *E. carotovora*. Two flasks were prepared with 100 μg/ml glycinol in 10 ml EMM. At the onset of the assay, one flask was inoculated with *E. carotovora* and both flasks incubated. At various times, 1.0-ml aliquots were removed from the flasks and each was extracted three times with 2 ml ethyl acetate. The ethyl acetate extracts of each aliquot were separately pooled and evaporated to dryness under N_2. The residues were resuspended in 50 μl ethyl acetate and 20-μl aliquots injected on the analytical HPLC column. Glycinol peak areas were compared to areas obtained by injection of glycinol standards and corrected for losses occurring during extraction. In the absence of bacteria, the amount of glycinol in the medium remains virtually constant over a 13-h incubation (Fig. 6). Therefore, glycinol does not decompose significantly in EMM. On the other hand, the glycinol concentration in the medium decreases in the presence of $\simeq 10^5$ CFU/ml *E. carotovora* (Fig. 6). Bacterial growth resumes approximately 11 h after inoculation, when the glycinol concentration in the medium has gone below 5 μg/ml (compare Figs. 5A and 6). The results presented here indicate that *E. carotovora* possesses the ability to detoxify glycinol allowing growth to resume.

A further experiment was performed to determine whether bacteria become tolerant to glycinol following exposure to the phytoalexin. An exponentially growing culture of *E. carotovora* ($\simeq 10^5$ CFU/ml) was treated with 50 μg/ml glycinol. The culture was incubated at 22 C until the glycinol-induced lag phase ended and exponential growth resumed (≃3.5 h). Addition of a second dose of glycinol (50 μg/ml) results in a second period of no growth (Fig. 7). The second inhibition period is reduced in length in inverse proportion to the number of bacteria in the culture (Fig. 7). This observation suggests that the ability of *E. carotovora* to recover from glycinol-induced growth inhibition is due to a constitutive rather than an inducible characteristic of the bacteria. If glycinol acts by blockage of a metabolic pathway and an alternate pathway is induced, then no second inhibition period should be observed. Alternatively, if the bacteria overcome the inhibitory effect of glycinol by induction of an enzyme which catalyzes the detoxification of glycinol, the duration of the second inhibition period would be expected to be significantly reduced beyond the reduction due to the increased population of bacteria. We believe the most likely explanation is that microbes possess a constitutive enzyme capable of detoxifying glycinol.

The inhibitory effect of glycinol can be overcome if a sufficiently high initial bacterial inoculum is used. Addition of a constant dose of glycinol (50 μg/ml) to cultures containing increasing numbers of *E. carotovora* (increasing optical densities) results in an inhibition period the length of which is inversely proportional to the number of bacteria in the culture (Fig. 8). A bacterial inoculum greater than 10^7 CFU/ml results in no observed inhibition in

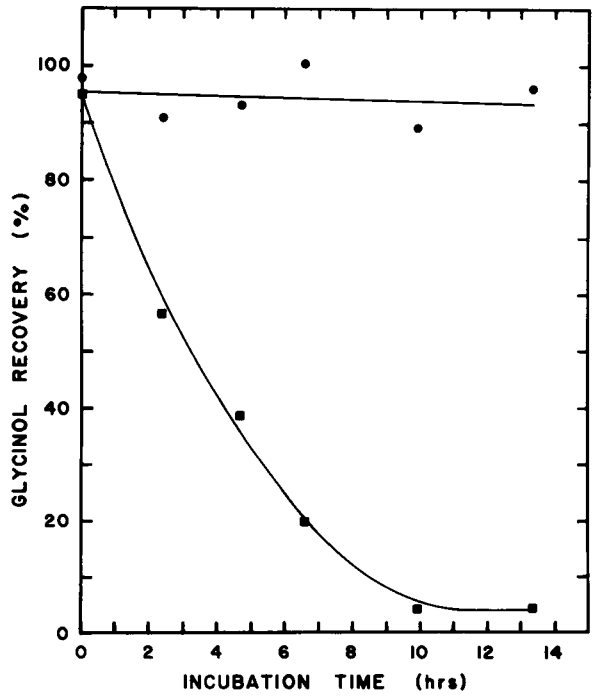

FIG. 6. Recovery of glycinol from EMM in the absence (●) and in the presence (■) of *E. carotovora*. Aliquots of the cultures (1.0 ml) were removed at various times, extracted with ethyl acetate, and the ethyl acetate soluble material analyzed for glycinol as described in the text.

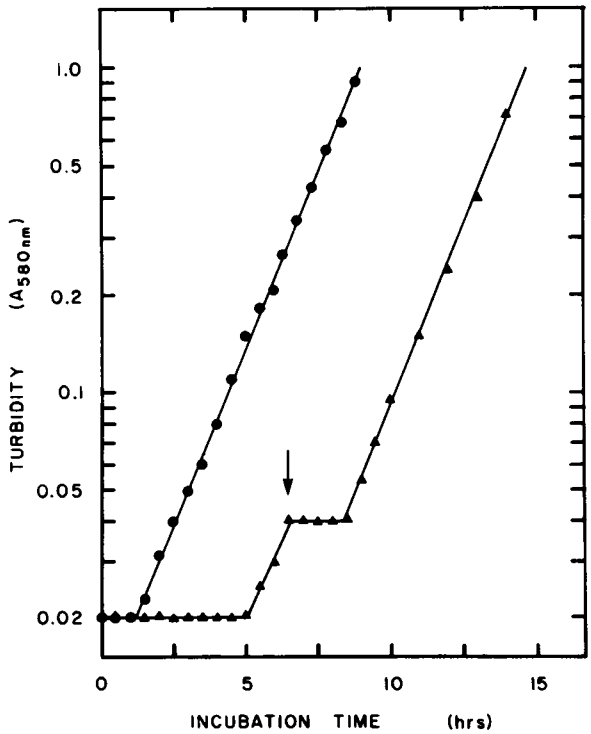

FIG. 7. Effect of successive additions of 250 μg glycinol, in 25 μl ethanol, to a culture (5 ml) of *E. carotovora* (▲). The first addition was made at 0 h; the arrow indicates the time of the second addition to the same culture. A control flask (●) received 25 μl ethanol at the onset of the experiment and at the arrow. Inoculations were carried out as described under "Materials and Methods."

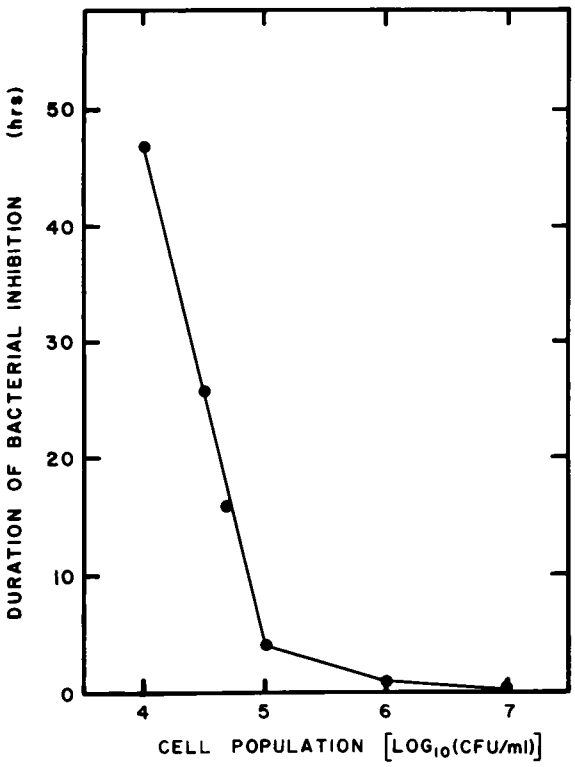

FIG. 8. Relationship between the duration of bacterial inhibition and the initial *E. carotovora* inoculum size in EMM containing 50 μg/ml glycinol. The duration of bacterial inhibition at each datum is the difference in the time required for a glycinol-inhibited culture and an uninhibited culture to resume exponential growth. Bacterial populations were determined by the number of colonies found upon dilution plating. Each datum represents the average of duplicate flasks.

medium containing 50 μg/ml glycinol (Fig. 8). This finding supports the hypothesis that microbes contain a constitutive enzyme which detoxifies glycinol.

A similar dependence of observed inhibitory activity upon initial inoculum of bacteria has been reported by Gnanamanickan and Patil (6) working with true bean (*Phaseolus vulgaris*) phytoalexins. An inoculum dependence on the duration of growth inhibition, like those observed for the antibacterial activities of glycinol and the true bean phytoalexins, could explain the contradictory reports of the ability of isoflavonoid phytoalexins to inhibit growth (7, 11, 19, 24). The inconsistencies in these studies are likely to be due to variations in the bacterial populations employed by the different workers in their *in vitro* assays. The use of sufficiently large initial inocula (Fig. 8) could therefore lead to the erroneous conclusion that the compound(s) in question has no antimicrobial activity.

The results reported in this paper show that glycinol is an antibiotic effective against a broad spectrum of microorganisms. Glycinol is a static agent which microorganisms can detoxify allowing growth to resume. A comparison of the antibacterial and antifungal activities of glycinol indicates that both procaryotes and eucaryotes are similarly affected by the phytoalexin *in vitro*. These results suggest that the ability of glycinol to inhibit growth is based on a similar mechanism of action in widely diverse microorganisms.

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