

Accumulation of isoflavonoids and isoflavone glucosides after inoculation of soybean leaves with *Xanthomonas campestris* pv. *glycines* and pv. *campestris* and a study of their role in resistance

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Pathogenic and nonpathogenic strains of *Xanthomonas campestris* pv. *glycines* (causal agent of bacterial pustule disease of soybean) or a strain of the soybean nonpathogen *X. campestris* pv. *campestris* were tested for their ability to grow and induce accumulation of isoflavonoids (glyceollin, coumestrol, daidzein, formononetin and genistein) and isoflavone glucosides (daidzin, genistin and ononin) in leaves of soybean cv. Clark (susceptible to bacterial pustule) and cv. Clark 63 (resistant to bacterial pustule due to the presence of the recessive *rpx* gene pair). Growth *in vivo* of nonpathogenic strains of pv. *glycines* and the strain of pv. *campestris* was highly restricted in cv. Clark while bacterial growth kinetics of a pathogenic strain of pv. *glycines* in the susceptible cv. Clark and in the resistant cv. Clark 63 were similar.

No or very low levels ($<50 \mu\text{g g}^{-1}$ fresh weight) of isoflavonoids including glyceollin accumulated by 4 days after inoculation of leaves of cvs Clark or Clark 63 with bacterial strains which showed restricted growth *in vivo*. In general, higher levels of isoflavone glucosides accumulated, but similar levels of isoflavone glucosides were found after inoculation with bacterial strains which were restricted or not restricted in their growth *in vivo*. In addition, the isoflavone glucosides were not inhibitory towards the xanthomonads in *in vitro* bioassays. Additional compounds of unknown identity with antibacterial activity also accumulated in certain interactions, but these compounds did not appear to be involved in resistance either. Resistance to pathogenic strains of pv. *glycines* conditioned by the *rpx* gene pair in cv. Clark 63 appears unusual in that bacterial growth is not restricted, but rather symptomology is reduced with a lowering of the number of pustular lesions accompanied by aborted growth and early necrosis of the pustules

INTRODUCTION

Bacterial pustule disease of soybean is incited by the bacterium *Xanthomonas campestris* pv. *glycines*. The disease is characterized by the formation of pustular lesions on infected leaves due to hypertrophy and possibly hyperplasia of host cells [19, 33, 42]. Under optimal conditions significant losses due to bacterial pustule can occur [16, 40]. Fortunately, resistance to this disease was recognized in the soybean cv. CNS over 30 years ago [17] and has been incorporated into many commercial cultivars. In the field this resistance is expressed as near immunity, with occasional small chlorotic spots, but no well defined pustules developing after inoculation with pv. *glycines* [17].

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The resistance is recessive and is due to a single major gene pair, the gene being designated *rpx* [17]. Resistance due to the *rpx* gene has not been overcome by pv. *glycines* and no physiologic races of this pathogen have been reported.

There have been few published attempts to determine the mechanism of resistance to bacterial pustule disease. Chamberlain [4] found similar growth of pv. *glycines* in leaf extracts from uninoculated resistant and susceptible leaves. The role of host peroxidases was assessed in a series of articles [6, 35, 36] and no conclusive evidence was found to support a role for peroxidases in resistance.

Fett & Sequeira [12] isolated, partially purified, and characterized a soybean seed glycoprotein which agglutinated cells of a pathogenic strain of pv. *glycines*. They hypothesized that attachment of this bacterium to the host cell wall via the bacterial agglutinin may be required for pathogenicity similar to the required attachment to host cell walls of *Agrobacterium tumefaciens* [31]. Resistance would be expressed if this agglutinin receptor site were lacking. However, this hypothesis was not supported when a large number of strains of pv. *glycines* were assayed against purified agglutinin from seed of both susceptible and resistant soybean cultivars [13].

Several isoflavonoids accumulate in soybean leaves after inoculation with incompatible phytopathogenic pseudomonads [22, 25]. Glyceollin (usually present as a mixture of isomers [3, 22]), daidzein and coumestrol were reported to increase to very high levels within 3 days of inoculation of soybean leaves with incompatible strains of *Pseudomonas syringae* pv. *glycinea* (causal agent of bacterial blight disease of soybean) [25]. The role of glyceollin in the restriction of bacterial growth has received the most critical assessment with current evidence indicating that its accumulation may lead to some reduction of growth, but that it is not the sole resistance mechanism [21].

Recently, our laboratory reported that isoflavone glucosides also accumulated in soybean leaves after inoculation with phytopathogenic pseudomonads [34].

Thus, we decided to assess the role of isoflavonoids and isoflavone glucosides in resistance of the susceptible soybean cv. Clark to both nonpathogenic strains of pv. *glycines* and to the nonsoybean (incompatible) pathogen *X. campestris* pv. *campestris* and their role in resistance of the near-isogenic highly resistant cv. Clark 63 (due to the *rpx* gene pair [41]) to pathogenic strains of pv. *glycines*. A preliminary report has been published [9].

MATERIALS AND METHODS

Bacterial strains

The source and the country or state of origin (when known) of bacterial strains are listed in Table 1.

Plant material and inoculation

Seed of soybean (*Glycine max*) cvs. Clark and Clark 63 were obtained from Dr R. L. Bernard, United States Regional Soybean Laboratory, Urbana, IL, USA. Seed of cabbage (*Brassica oleracea capitata*) cv. Golden Acre was purchased from Olds Seed Company*, Madison, WI, USA.

*Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

TABLE 1
Source and origin of bacterial strains

Bacterium	Strain	Source	Country or state of origin
<i>Erwinia chrysanthemi</i>	SR 239	A. Kelman	Italy
<i>Proteus vulgaris</i>	13315	ATCC ^a	—
<i>Pseudomonas solanacearum</i>	B1	L. Sequeira	—
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	Xc42	UWCC ^b	—
	Xc43	UWCC	—
pv. <i>glycines</i>	1717	NCPPB ^c	Zimbabwe
	XP175	M. P. Starr	Sudan
	A	ERRC ^d	—
	S-9-4	W. Fett	Wisconsin
	1714	NCPPB	Zambia
	1716	NCPPB	Zambia
	1136	NCPPB	Zimbabwe
	S-9-8	W. Fett	Wisconsin

^aATCC = American Type Culture Collection, Rockville, MD, USA.

^bUWCC = University of Wisconsin Culture Collection, Department of Plant Pathology, Madison, WI, USA.

^cNCPPB = National Collection of Plant Pathogenic Bacteria, Hatching Green, England.

^dERRC = Eastern Regional Research Center, Philadelphia, PA, USA.

Cabbage and soybean seed were sown in Baccto potting soil (Michigan Peat Company, Houston, TX, USA) contained in clay pots. All plants were grown in a growth chamber maintained at 24 °C day, 20 °C night, 75% RH, with fluorescent and incandescent bulbs providing 1.1×10^4 lx during a 13-h photoperiod.

To prepare inocula, bacterial cells grown on nutrient agar (NA) (Difco Laboratories, Detroit, MI, USA) for 24 to 48 h at 28 °C were suspended in sterile water and adjusted turbidimetrically to a final concentration of approximately 10^8 colony-forming units (cfu) ml⁻¹ as determined by reference to a standard curve of OD_{600 nm} vs cfu ml⁻¹ generated by dilution plating techniques. When required, dilutions of the standard inocula were prepared with sterile water. When heat-killed bacteria were to be used for inoculation, bacterial suspensions were heated in a boiling water bath for 20 to 30 min. Aliquots (0.1 ml) of the heated suspensions were plated onto NA. No bacterial growth occurred.

Soybean plants had one or two trifoliolate leaves when used for inoculation. Only the uppermost fully-opened, but not yet fully-expanded, trifoliolate leaf on each plant was inoculated. Plants were inoculated by forcibly spraying bacterial suspensions against the abaxial side of the leaflets until the tissue appeared water-soaked [14, 27]. When bacterial growth *in vivo* and accumulation of isoflavonoids and isoflavone glucosides were to be followed simultaneously, spraying continued until approximately 90% of the abaxial leaf surface had a water-soaked appearance. Inoculated leaves were rinsed well in tap water and plants were set on the lab bench until all appearance of water-soaking disappeared (1 to 2 h). Plants were then returned to the growth chamber and the chamber temperature was adjusted upwards to 28 °C day, 24 °C night.

To test for pathogenicity of *pv. campestris* strains, undiluted inocula were either injected into leaf veins or into interveinal areas of 6-week-old cabbage plants. Inoculated plants were returned to the growth chamber and were observed for symptoms for up to 9 days after inoculation.

Biochemical and physiological tests

Ability of bacterial strains to grow at 35 °C was tested by inoculating tubes containing a yeast-salts broth [7] and incubating in a temperature-controlled shaking water bath. Growth was monitored visually over a 7-day period.

To test for esculin hydrolysis, tubes containing the broth medium of Sneath [37] were inoculated and incubated at 28 °C.

Bacteria were tested for mucoid growth on nutrient dextrose agar (Difco NA plus 10 g l⁻¹, Difco dextrose and 5 g l⁻¹, Difco yeast extract). Agar plates were streaked with bacteria and incubated at 28 °C.

Gelatin liquefaction was tested using NA supplemented with 0.4% gelatin [38]. Inoculated plates were incubated at 28 °C for 72 h.

Hydrolysis of casein was tested using NA containing Difco skim milk (50%, v/v). Inoculated plates were incubated at 28 °C for up to 14 days.

The ability to produce urease was determined on Christensen urea agar [5]. Inoculated plates were incubated at 28 °C for up to 72 h. *Proteus vulgaris* strain 13315 was included as a positive control organism.

Tolerance to NaCl was tested using the NaCl containing broth described by Hayward [18]. Inoculated tubes were incubated at 28 °C for 7 days.

Acid production from various carbohydrates was tested using the basal medium of Hayward [18]. All carbohydrates were filter-sterilized before use. L(+)-arabinose, D-mannose and D(+)-galactose were purchased from Sigma Chemical Company, St Louis, MO, USA; α,α' -D-trehalose dihydrate and D-cellobiose from Pierce Chemical Company, Rockford, IL, USA; D-glucose (Bacto dextrose) from Difco Laboratories. Stab inoculated culture tubes were incubated at 28 °C.

Motility was checked by observing wet mounts under phase microscopy at 600 \times . Motility was also determined by placing the inoculum into a piece of open-ended glass tubing which extended above the surface of a semisolid medium [Difco nutrient broth plus 0.4% (w/v) of Difco Bacto agar] [30]. Tubes were incubated at 28 °C for up to 18 days.

Lecithinase (phospholipase) and lipase activities were detected using egg yolk agar [20]. Inoculated plates were incubated at 28 °C for up to 7 days. *Erwinia chrysanthemi* strain SR 239 was used as a positive control organism for lecithinase production.

Bacteria were tested for their ability to grow on SX agar [37], a medium which differentiates certain *X. campestris* pathovars. SX agar was prepared as described [37] except that the cyclohexamide was omitted. Bacteria were either streaked heavily onto the agar surface or dilutions, prepared from bacterial suspensions in sterile water ($OD_{600\text{ nm}} = 0.10$), were surface-plated to give single colony growth.

Induction of the hypersensitive response (HR) on tobacco (*Nicotiana tabacum* cv. Bottom Special) was tested using the method of Klement [29]. Sterile water sus-

pensions containing approximately 10^9 cfu ml⁻¹ were injected into leaf intercostal areas. *Pseudomonas solanacearum* strain B1 was used as a positive control organism.

The pectate agar medium of Beraha [2] was used to test for pectolytic activity. Polygalacturonic acid (Grade II, Sigma Chemical Company) was used and the pH of the medium adjusted to pH 5.0 or 8.5 before sterilization. Media pH differed only by ± 0.1 pH unit after sterilization. Pectate agar plates were stab inoculated and then incubated at 28 °C.

Bacterial growth in vivo and accumulation of isoflavonoids and isoflavone glucosides

Bacterial growth *in vivo* was determined as follows. For each sampling time and bacterial strain duplicate sets of three leaf discs (1 cm² diam.) from a single inoculated plant (one disc per leaflet) were removed from the inoculated areas. Each sample consisting of three leaf discs was triturated in 2 ml of 0.01 M phosphate buffer, pH 6.8 and the number of bacteria present determined by standard dilution plating techniques.

For each bacterial strain and sampling time the leaf material remaining after removal of leaf discs for bacterial growth determinations was combined and plunged into boiling 80% aqueous ethanol (EtOH) for 10 min to extract isoflavonoids and isoflavone glucosides and to inactivate any glucosidases present. After removal of leaf residue by filtration, filtrates were evaporated to dryness under a stream of nitrogen and each sample partitioned between diethyl ether:30% aqueous EtOH (2:1) [34].

Quantitation of isoflavonoids and isoflavone glucosides

Commercial samples of coumestrol (Eastman Kodak Company, Rochester, NY, USA), daidzein, formononetin and genistein (ICN Pharmaceuticals, Plainview, NY, USA) were obtained. Coumestrol was further purified by recrystallization from EtOH-water. Mixtures of glyceollin isomers were obtained from either soybean seed sprayed with *Cladosporium* sp. spores [11] or leaves of cv. Harosoy inoculated with an incompatible strain of *P. syringae* pv. *glycinea*. Glyceollin from seed was purified as described by Fett & Osman [11] except that silica gel column chromatography was substituted for preparative silica gel TLC. Glyceollin from soybean leaves was extracted by a facilitated diffusion technique [23]. Purification of leaf glyceollin was achieved by preparative silica gel TLC followed by HPLC as described below.

Purified standards of daidzin, genistin and ononin were obtained as described by Osman & Fett [34].

Glyceollin concentration was determined on the basis of dry weight and confirmed by measuring the absorption at 285 nm and using a molar extinction coefficient in ethanol of 10 300 [1]. Concentration of the remaining standards was determined gravimetrically.

Crude leaf extracts (200 mg fresh weight equivalents) were subjected to TLC as described previously [34]. Areas corresponding to R_f of purified standards run on the same plate were removed. The zones corresponding to daidzin, genistin and ononin were scraped off together, as was the zone corresponding to genistein, formononetin and coumestrol standards. Daidzein and glyceollin areas were scraped off separately. Quantification was accomplished by HPLC with a Waters Associates

(Milford, MA, USA) system consisting of a Model U6K injector, a Model M-6000A chromatography pump, and a Model 450 variable wavelength detector. The aqueous EtOH-soluble extracts were analysed on a C₁₈ μ m Bondapak column (10 μ m, 3.9 i.d. \times 30 cm) (Waters Associates) eluted with 40% aqueous methanol (MeOH). Absorbance at 260 nm was monitored. Daidzin, genistin and ononin had elution times of approximately 3.8, 6.4 and 12.4 min, respectively. Quantification was done by comparison of peak areas for leaf extracts with these for pure standards of known concentration.

Reverse-phase HPLC was also used when the genistein content of diethyl ether-soluble leaf extracts was to be determined. High-performance liquid chromatography was carried out as described for the aqueous EtOH soluble leaf extracts except 50% aqueous MeOH was used for elution. Genistein, formononetin and coumestrol standards had elution times of approximately 10.2, 17.6 and 18.7 min, respectively.

Diethyl ether-soluble fractions were analysed for glyceollin using a μ m Porasil column (10 μ m, 3.9 i.d. \times 30 cm) (Waters Associates) eluted with 5% isopropanol in hexane. Absorbance at 285 nm was monitored. Glyceollin isomers I, II and III had elution times of approximately 7.5, 7.9 and 8.3 min, respectively. Analyses for daidzein, formononetin and coumestrol were run as described for glyceollin except that 7% isopropanol in hexane was used for elution. Absorbance at 260 nm was monitored. Formononetin, coumestrol and daidzein had retention times of approximately 5.0, 6.6 and 7.8 min, respectively. Normal phase HPLC could not be used for quantification of genistein since reproducible peak areas for genistein standards were not obtained.

Recovery of glyceollin and daidzin standards added to crude leaf extracts before partitioning between diethyl ether and 30% aqueous EtOH was approximately 60% [34]. Further confirmation of the identity of isoflavonoids and isoflavone glucosides from soybean leaves was accomplished by UV spectroscopy and GC/MS as described previously [34].

Bioassays for presence of antifungal and/or antibacterial compounds

The presence of antifungal compounds in all crude aqueous EtOH or diethyl ether-soluble leaf extracts (before subjecting to TLC) was tested by the *Cladosporium* sp. TLC bioassay [26]. Twenty-five to 100 μ l of crude leaf extracts (corresponding to 25 to 100 mg fresh weight of leaf tissue) were used for spotting Silica gel GF, 250 μ TLC plates. Appropriate standard compounds (2 μ g each) were also spotted onto each TLC bioassay plate. Plates were developed either in cyclohexane:EtOAc (1:1) (for diethyl ether-soluble extracts) or [CHCl₃:(CH₃)₂CO:MeOH (20:6:5)] (for aqueous EtOH-soluble extracts).

The presence of antibacterial compounds in crude aqueous EtOH or diethyl ether-soluble leaf extracts was also tested using a TLC bioassay which utilized the ability of both pv. *glycines* and pv. *campestris* strains to hydrolyse esculin to give a yellow coloured product [32]. Silica gel GF, 250 μ TLC plates were spotted with 100 μ l (corresponding to 100 mg fresh weight of leaf tissue) of crude leaf extracts. Appropriate standard compounds (25 μ g of glyceollin and 2 μ g of the other standards) also were spotted on each TLC bioassay plate. Plates were developed in the appropriate solvent (see above), left to air-dry, and then sprayed with bacterial suspensions

($OD_{600\text{ nm}} = 1.0$) prepared in trypticase soybroth (BBL, Cockeysville, MD, USA). On each silica gel TLC plate, only one leaf extract obtained from leaves inoculated with bacteria plus an appropriate control extract from leaves sprayed with sterile water alone were tested, and the plate was sprayed with a bacterial suspension prepared with the same bacteria as used for the leaf inoculation. After 20 to 24 h incubation at room temperature under 100% RH, plates were resprayed with an esculin-containing broth [32]. Within 1 to 2 h a strong yellow background developed on the plates due to the presence of metabolically-active bacteria. The presence of antibacterial compounds was indicated by a white area (due to silica gel) on the yellow background. Thin-layer chromatography plates not spotted with leaf extracts and sprayed with TSB alone were also sprayed with esculin broth as a control.

Purified compounds were tested for antibacterial activity using the direct spotting bioassay method of Wyman & Van Etten [43] as described previously [11]. Glyceollin obtained from leaves inoculated with bacteria was used in this assay. Leaf glyceollin is predominantly isomer III [11, 22]. Glyceollin was prepared at 2 mg ml^{-1} and daidzein and formononetin at 1 mg ml^{-1} in 95% EtOH. Coumestrol was prepared at 2 mg ml^{-1} in dimethyl sulphoxide (DMSO) (Fischer Scientific, Fair Lawn, NJ 07410), and daidzin, genistin and ononin at 1 mg ml^{-1} in MeOH. For all test compounds volumes of $5\text{ }\mu\text{l}$ were spotted. After the solvents evaporated, only formononetin and coumestrol left a visible residue on the agar surface. Streptomycin sulphate (Boehringer Mannheim, West Germany) at 2 mg ml^{-1} in water was spotted ($2\text{ }\mu\text{l}$) as a positive control. Plates were incubated for 48 h at $28\text{ }^{\circ}\text{C}$.

RESULTS

Pathogenicity tests

For pathogenicity tests of pv. *glycines* strains, inocula were diluted 1:10 or 1:1000 with sterile water to give final bacterial concentrations of approximately 10^7 or 10^5 cfu ml^{-1} , respectively. Numerous pustular lesions typical of bacterial pustule disease of soybean [28] were present on leaves of cv. Clark by 3 to 4 days after inoculation with pv. *glycines* strains XP175, A, S-9-4 or 1717. In this time period strain 1714 induced both browning of the leaf laminae (primarily visible on the abaxial side) and a low number of pustular lesions. When inocula contained 10^5 cfu ml^{-1} strains S-9-8, 1136 or 1716 did not cause pustular lesion formation or browning of the leaf laminae by 7 days after inoculation. When inocula contained 10^7 cfu ml^{-1} , strains S-9-8, 1136 and 1716 induced either no symptoms or slight browning of leaf laminae evident by 3 days after inoculation.

Both pv. *campestris* strains Xc42 and Xc43 were pathogenic on cabbage cv. Golden Acre. Symptoms consisted of blackening of leaf veins plus spreading chlorotic lesions on leaf laminae which later turned necrotic.

Biochemical and physiological tests

Three pathogenic strains of pv. *glycines* (1717, XP175 and A) and the three non-pathogenic strains (S-9-8, 1716 and 1136) were used in all tests, except in determination of the ability to induce the HR in tobacco leaves. For this test, strains XP175, S-9-4, S-9-8 and 1716 were used. The pathogenic and nonpathogenic strains of pv. *glycines*

all gave reactions typical for *X. campestris* [8]. All strains were motile, positive for lipase, and negative for both lecithinase and induction of an HR in tobacco. Only subtle differences between pathogenic and nonpathogenic strains were found. Four of the six strains of pv. *glycines* could grow in NaCl broth containing up to 3% NaCl. Pathogenic strain XP175 could only tolerate up to 2% NaCl and nonpathogenic strain 1136 could tolerate up to 4% NaCl. Nonpathogenic pv. *glycines* strains demonstrated higher pectolytic activity than pathogenic strains when tested at pH 5.0 and 8.5. Nonpathogenic strains grew more rapidly than pathogenic strains and caused visible pitting of the pectate agar medium within 24 h of incubation, compared to 48 h for pathogenic strains.

Plating efficiencies on SX agar media were quite variable between pv. *glycines* strains ranging from 0 to 95%. There was no relationship between pathogenicity of the pv. *glycines* strains and their ability to grow on SX agar. Of the six strains tested, only one strain, XP175, could not grow as single colonies even when plated from a bacterial suspension containing approximately 10^6 cfu ml⁻¹. This strain did grow on SX agar when a loopful of bacteria taken off an agar culture of the bacterium was streaked across the agar surface. Pathogenic and nonpathogenic pv. *glycines* strains could be differentiated by their appearance on SX agar after 5 days incubation. Colonies produced by nonpathogenic strains had blue to purple centres with no greenish hue, while colonies of pathogenic pv. *glycines* strains had light purple centres and a definite greenish hue.

Bacterial growth in vivo and stress metabolite accumulation

Preliminary experiments indicated that soybean plants grown under our growth chamber conditions were free of levels of microbes that would necessitate the use of antibiotic resistant bacterial mutants for *in vivo* growth experiments. Undiluted bacterial inocula was used in experiments designed to follow both bacterial growth and accumulation of isoflavonoids and isoflavone glucosides simultaneously.

When leaves of cv. Clark were inoculated with suspensions of pathogenic pv. *glycines* strains XP175, A, 1717 and S-9-4 containing approximately 10^8 cfu ml⁻¹, the first symptom observed was a light green chlorosis of the inoculated area visible on the adaxial leaf surface by 2 days after inoculation. By 3 days after inoculation a strong yellowing of the leaf laminae occurred primarily visible on the adaxial leaf surface with pustular lesions present only at the edges of the inoculated area on the abaxial leaf surface. Some leaflets inoculated with pathogenic strains of pv. *glycines* abscised between 3 and 4 days after inoculation. Strain 1714 induced both pustular lesions plus moderate browning and yellowing in the inoculated area by 3 days after inoculation. Heat-killed cells of strain 1714 induced no symptoms by 4 days after inoculation. Inoculation with the nonpathogenic pv. *glycines* strain 1716 at this high inoculum concentration caused strong browning of the leaf laminae in the inoculated areas which was visible within 2 days after inoculation. Nonpathogenic pv. *glycines* strains S-9-8 and 1136 and the incompatible pv. *campestris* strain Xc42 induced slight browning of the laminae (primarily visible on the abaxial surface) and light green chlorosis (primarily visible on the adaxial surface) by 2 days after inoculation. Browning and chlorosis had not increased in intensity 4 days after inoculation. None

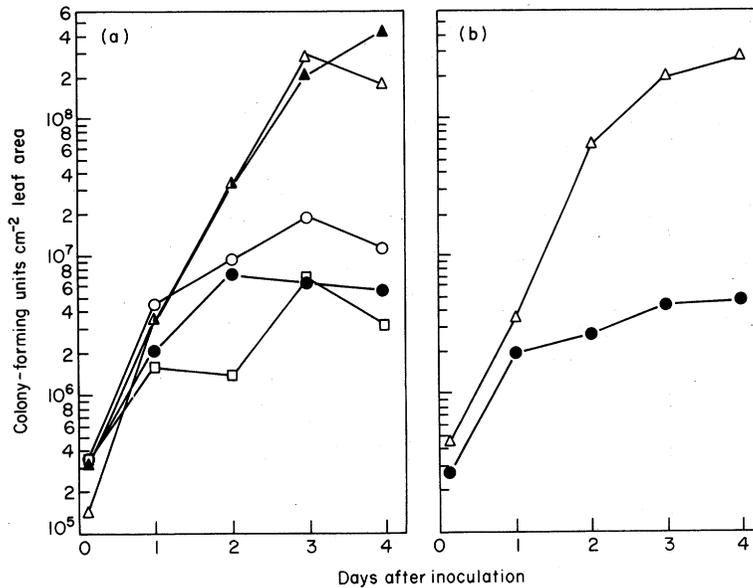


FIG. 1. Bacterial growth in trifoliolate leaves of soybean (a) cv. Clark and (b) cv. Clark 63 using bacterial suspensions containing approximately 10^8 cfu ml⁻¹ for inoculation. Δ , *Xanthomonas campestris* pv. *glycines* strain XP175; \blacktriangle , pv. *glycines* strain A; \circ pv. *glycines* strain 1716; \bullet , pv. *glycines* strain S-9-8; \square , pv. *campestris* strain Xc42. Values represent averages of data from two separate experiments.

of the three nonpathogenic strains of pv. *glycines* caused pustular lesion formation or abscission of inoculated leaflets by 4 days after inoculation.

When leaves of resistant cv. Clark 63 were inoculated with bacterial suspensions containing 10^8 cfu ml⁻¹, pathogenic pv. *glycines* strain XP175 induced light green chlorosis and slight browning by 2 days after inoculation. Browning increased in intensity thereafter and chlorosis increased in intensity to severe yellowing by 3 days after inoculation. As with the cv. Clark-strain XP175 interaction, leaf abscission sometimes occurred between 3 and 4 days after inoculation. No pustules were formed by 4 days after inoculation. Over the 4-day incubation period nonpathogenic pv. *glycines* strain S-9-8 induced only slight browning and light green chlorosis, first evident by 2 days after inoculation.

The maximum bacterial populations achieved by nonpathogenic pv. *glycines* strains 1716 and S-9-8 and by the incompatible pv. *campestris* strain Xc42 (7×10^6 to 2×10^7 cfu cm⁻² leaf surface area) in leaves of cv. Clark were reduced compared to those achieved by the pathogenic pv. *glycines* strains XP175 and A (2.9 to 4.3×10^8 cfu cm⁻² leaf surface area) [Fig. 1(a)]. All bacterial strains tested showed similar growth in the first 24 h after inoculation, but thereafter logarithmic growth continued for the pathogenic strains of pv. *glycines* for the next 2 days, while growth of the nonpathogenic strains of pv. *glycines* and the incompatible strain of pv. *campestris* was restricted. Growth of pv. *glycines* strains 1717, S-9-4, 1714 and 1136 in leaves of cv. Clark was not determined.

Bacterial growth kinetics for pv. *glycines* strains XP175 and S-9-8 in leaves of

TABLE 2
Accumulation of isoflavonoids and isoflavone glucosides in leaves of susceptible soybean cv. Clark or resistant cv. Clark 63 3 days after inoculation with bacteria

Cultivar-bacterium interaction	Bacterial strain	Isoflavonoids and isoflavone glucosides ($\mu\text{g g}^{-1}$ fresh weight)							
		Glyceollin	Coumestrol	Daidzein	Formononetin	Genistein	Daidzin	Ononin	Genistin
cv. Clark									
<i>X. campestris</i>									
<i>pv. campestris</i>	Xc42	2 \pm 1 ^b	...	35 \pm 8	31 \pm 8	41 \pm 17
<i>pv. glycines</i>	XP175	38 \pm 13	2 \pm 3	2 \pm 2	2 \pm 1	NT ^c	67 \pm 41	7 \pm 1	46 \pm 8
	A	TR ^d	32 \pm 6	4 \pm 2	62 \pm 30
	S-9-4	...	NT	NT	NT	NT	23 \pm 28	NT	NT
	1717	...	NT	NT	NT	NT	72 \pm 92	NT	NT
	1714	276 \pm 9	NT	NT	NT	NT	123 \pm 21	NT	NT
	1714 (heat-killed)	...	NT	NT	NT	NT	TR	NT	NT
	S-9-8	TR	TR	NT	3 \pm 1	...	3 \pm 1
	1136	5 \pm 7	NT	NT	NT	NT	41 \pm 56	NT	NT
	1716	8 \pm 6	35 \pm 15	8 \pm 1	18 \pm 4	TR	35 \pm 1	9 \pm 1	14 \pm 7
Sterile water		NT	4 \pm 7
cv. Clark 63									
<i>pv. glycines</i>	XP175	76 \pm 83	5 \pm 3	18 \pm 21	7 \pm 1	NT	113 \pm 60	9 \pm 3	63 \pm 18
	S-9-8	...	TR	2 \pm 2	1 \pm 1	NT	12 \pm 4	5 \pm 1	16 \pm 13
Sterile water		4 \pm 6	NT	TR	...	TR

^a(...) = none detected.

^bValues \pm standard deviation and are uncorrected for extraction efficiency.

^cNT = not tested.

^dTR = trace ($<1.0 \mu\text{g g}^{-1}$ fresh weight).

resistant cv. Clark 63 [Fig. 1(b)] were similar to those found for these strains on the susceptible cv. Clark [Fig. 1(a)].

In leaves of the susceptible cv. Clark only pv. *glycines* strains XP175 (pathogenic), 1714 (intermediate in pathogenicity) and 1716 (nonpathogenic) induced accumulation of appreciable levels of isoflavonoids (Table 2). Strains XP175 and 1714 were the most efficient inducers of glyceollin accumulation and strain 1716 of daidzein, formononetin and coumestrol. The incompatible pv. *campestris* strain Xc42 only induced accumulation of formononetin and only to low levels. Heat-killed cells of pv. *glycines* strain 1714 did not induce glyceollin accumulation (Table 2). Only two strains of pv. *glycines* were tested for their ability to induce accumulation of isoflavonoids in leaves of resistant cv. Clark 63. The pathogenic strain XP175, but not nonpathogenic pv. *glycines* strain S-9-8, induced accumulation (Table 2). The levels of isoflavonoids induced by pv. *glycines* strain XP175 in leaves of the resistant cv. Clark 63 were similar to those induced in susceptible cv. Clark (Table 2). Genistein accumulation was determined only for the interaction of cv. Clark with pv. *glycines* strains A and 1716 and pv. *campestris* strain Xc42. Genistein did not accumulate in any of these interactions.

All bacterial strains tested induced accumulation of glucosides in leaves of cvs Clark and Clark 63 (Table 2) except for the nonpathogenic pv. *glycines* strain S-9-8 which did not do so in cv. Clark. Heat-killed cells of pv. *glycines* strain 1714 did not induce accumulation of daidzin (Table 2).

In interactions of cvs Clark and Clark 63 with bacteria where isoflavonoids and/or isoflavone glucosides accumulated, accumulation was usually first detected 2 days after inoculation (data not shown). This coincided with the onset of restricted growth for nonpathogenic pv. *glycines* and incompatible pv. *campestris* strains, but pathogenic pv. *glycines* strains were still demonstrating logarithmic growth [Fig. 1(a)]. Kinetics of accumulation of these compounds were erratic with values increasing or decreasing between 2 and 4 days after inoculation (data not shown).

Since we did not find reduced growth of pathogenic pv. *glycines* strain XP175 in the resistant cv. Clark 63 when compared to growth in the susceptible cv. Clark (Fig. 1), and since the use of such high bacterial concentrations in inocula inhibited formation of pustular lesions in the susceptible cv. Clark, a further set of experiments was run which followed growth and pustule formation of pv. *glycines* strain XP175 in leaves of these two cultivars inoculated with a lower initial inoculum concentration (standard inoculum diluted 1 : 100 to give approximately 10^6 cfu ml⁻¹). Results showed that again growth of pv. *glycines* strain XP175 was not restricted in cv. Clark 63 (Fig. 2). However, differences in symptomology on the two cultivars were noted. Symptoms were observed both with the naked eye and with the aid of a binocular microscope at up to 64× magnification. Pustules were first evident on cv. Clark by 3 to 4 days after inoculation. By 7 days after inoculation the pustules had greatly enlarged and some appeared ruptured but not necrotic. By 9 days some pustules were slightly browned. On cv. Clark 63 pustule formation also occurred but pustules were not evident until 4 to 5 days after inoculation. By 7 days after inoculation pustules appeared much smaller on the average and were less numerous than those present at this time on cv. Clark. Pustules were browned and lesions necrotic. By 9 days pustular lesions were black. Moderate yellowing of the inoculated tissue was evident by 7 days after inoculation of both cultivars but no leaf abscission occurred.

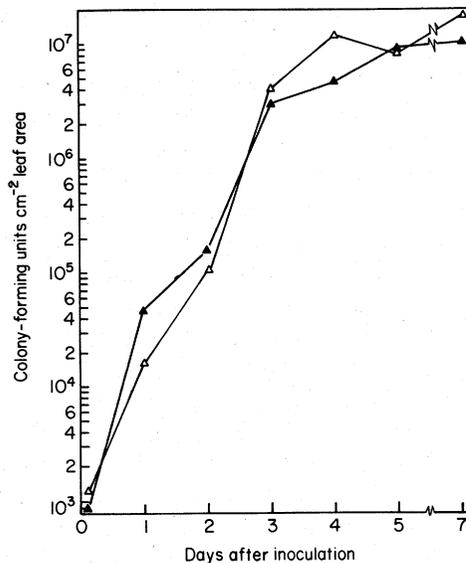


FIG. 2. Growth of *Xanthomonas campestris* pv. *glycines* strain XP175 in trifoliolate leaves of susceptible or resistant soybean cultivars using bacterial suspensions containing approximately 10^8 cfu ml⁻¹ for inoculation. Δ , cv. Clark; \blacktriangle , cv. Clark 63. Values represent averages of data from two separate experiments.

Thin-layer chromatographic bioassays for antifungal and antibacterial compounds in crude leaf extracts

Thin-layer chromatography bioassays of crude diethyl ether-soluble leaf extracts from the interaction of cv. Clark with pv. *glycines* strains XP175 (pathogenic), 1714 (intermediate in pathogenicity) and 1716 (nonpathogenic), and of cv. Clark 63 with strain XP175, consistently demonstrated the presence of two areas of inhibition centred at $R_F = 0.67$ and 0.55 with a third area centred at $R_F = 0.39$ occasionally present (Table 3). The areas of antifungal activity corresponded in R_F to the areas of antibacterial activity. The R_F values of the three areas of inhibition were similar to standards of glyceollin (0.67), formononetin (0.55) and coumestrol (0.52), and daidzein (0.39). Heat-killed cells of pv. *glycines* strain 1714 did not induce any diethyl ether-soluble inhibitory compounds. Thin-layer chromatography bioassays of crude diethyl ether-soluble leaf extracts from the interactions of pv. *glycines* strains 1717 (pathogenic), 1136 and S-9-8 (both nonpathogenic) with cv. Clark occasionally demonstrated the presence of slight areas of inhibition of the *Cladosporium* sp., but there was no evidence for the presence of antibacterial compounds (Table 3). Crude diethyl ether-soluble leaf extracts from the interactions of the remaining strains of pv. *glycines* or pv. *campestris* did not show antifungal or antibacterial activity. The compound responsible for the inhibitory zone at $R_F = 0.67$ was glyceollin. The glyceollin standards run on each TLC bioassay plate always had the same R_F as this inhibitory zone and were antifungal and antibacterial. Glyceollin is known to be highly inhibitory towards *Cladosporium* sp. [11, 26] and is inhibitory towards pv.

TABLE 3
 Presence of antifungal and antibacterial activities in crude extracts obtained from bacterially-inoculated soybean leaves 3 days after inoculation as determined by TLC bioassays

Cultivar-pathovar interaction	Bacterial strain	Diethyl ether-soluble ^a		Aqueous ethanol-soluble ^b	
		R _F of antifungal spots ^c	R _F of bacterial spots ^d	R _F of anti-fungal spots	R _F of anti-bacterial spots
Clark— <i>Xanthomonas campestris</i>					
pv. <i>campestris</i>	Xc42	... ^e	...	± 0.88	...
pv. <i>glycines</i>	1717	± 0.55	...	± 0.88	...
	XP175	0.67, 0.55, ± 0.39	0.67, 0.55	0.88	0.88
	A
	S-9-4
	1714	0.67, 0.55	0.67, 0.55, ± 0.39	0.88	± 0.88
	1714 (heat-killed)
	1716	0.67, 0.55	0.55	0.88	0.88
	1136	± 0.55
	S-9-8	± 0.67, ± 0.39
Sterile water	—
Clark 63—					
pv. <i>glycines</i>	XP175	0.67, 0.55	0.67, 0.55	0.88	0.88
	S-9-8
Sterile water	—

^aR_F values were 0.67, 0.55, 0.52 and 0.39 for standards of glyceollin, formononetin, coumestrol and daidzein, respectively.

^bR_F values were 0.51, 0.45 and 0.41 for standards of ononin, genistin and daidzin, respectively.

^cActivity against *Cladosporium* sp.

^dActivity against the same bacterial strain as was used for inoculation of leaves.

^e(...) = none detected.

glycines [11, 25, see below] and pv. *campestris* [11, see below]. The compounds responsible for the areas of inhibition centred at R_F = 0.55 and 0.39 were not formononetin, coumestrol or daidzein even though their mobilities were similar. This was indicated by the lack of antifungal activity when purified standards of formononetin, daidzein (both tested up to 200 µg) and coumestrol (tested up to 50 µg) were assayed using the TLC bioassay and the lack of inhibitory activity of formononetin and daidzein standards against pv. *glycines* and pv. *campestris* (see below). In addition, areas of antibacterial activity found for the interaction of cv. Clark with pathogenic pv. *glycines* strain XP175 centred at R_F = 0.55 were as large or larger than those found for the interaction of cv. Clark with nonpathogenic pv. *glycines* strain 1716 even though much higher levels of coumestrol accumulated in the latter interaction.

Thin-layer chromatography bioassays of crude aqueous EtOH-soluble leaf extracts from the interactions of cv. Clark with pv. *glycines* strains XP175, 1714 and 1716 and of cv. Clark 63 with strain XP175, consistently demonstrated the presence of one

TABLE 4

Antibacterial activity of isoflavonoids and isoflavone glucosides as determined by direct spotting onto agar medium seeded with bacteria

Bacterium	Strain	Glyceollins (10 µg)	Daidzein (5 µg)	Daidzin (5 µg)	Formononetin (5 µg)	Ononin (5 µg)	Coumestrol (10 µg)	Genistin (5 µg)
<i>Xanthomonas campestris</i> <i>pv. glycines</i>	S-9-8	2.0 ± 0.0 ^a	0	0	0	0	0	0
	1136	1.5 ± 0.6	0	0	0	0	0	0
	1716	2.0 ± 0.0	0	0	0	0	0.5 ± 0.6	0
	A	2.5 ± 1.0	0	0	0	0	0	0
	XP175	2.8 ± 0.5	0.2 ± 0.4	0	0	0	0.8 ± 0.8	0
	1717	2.0 ± 0.0	0	ND ^b	0	ND	1.0 ± 0.0	ND
	<i>pv. campestris</i>	Xc42	1.7 ± 1.2	0	0	0	0	1.0 ± 0.0
	Xc43	2.5 ± 0.6	0	0	0	0	1.3 ± 0.5	0

^aRating 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 ± standard deviation.

^bND = not determined.

antifungal and antibacterial compound with $R_f = 0.88$ (Table 3). When observed under 254 nm light this area of the plate contained a blue fluorescent compound, but whether or not this was the active compound has not been established. Thin-layer chromatography bioassays of crude aqueous EtOH-soluble leaf extracts from all other interactions including heat-killed cells of pv. *glycines* strain 1714 with cv. Clark demonstrated the presence of only trace amounts of this compound(s) or no antifungal or antibacterial compounds (Table 3).

Direct spotting bioassay of purified compounds

Of the purified compounds tested for antibacterial activity using the direct spotting bioassay, only the mixture of glyceollin isomers (predominating in isomer III) had moderate activity against the bacteria tested (Table 4). In the instances where inhibition by 95% EtOH or DMSO alone occurred (never more than a trace of inhibition) the value was subtracted before inclusion in Table 4.

DISCUSSION

Certain strains of *X. campestris* pv. *glycines* and the one strain of *X. campestris* pv. *campestris* tested were capable of inducing accumulation of low levels of soybean isoflavonoids, and all bacterial strains induced accumulation of isoflavone glucosides. It does not appear, however, that bacterially-induced accumulation of isoflavonoids and/or isoflavone glucosides is responsible for resistance of either the susceptible cv. Clark to nonpathogenic strains of pv. *glycines* or to the incompatible strain of pv. *campestris*, or of the resistant cv. Clark 63 to pathogenic and nonpathogenic strains of pv. *glycines*.

Of the purified isoflavonoids and isoflavone glucosides tested in our bioassays, only glyceollin showed significant inhibitory activity against pv. *glycines* and pv. *campestris*. Glyceollin either did not accumulate or accumulated to very low levels during the interaction of nonpathogenic strains of pv. *glycines* and incompatible pv. *campestris* with cvs Clark and Clark 63, even though *in vivo* growth of these strains was restricted. Glyceollin accumulated to higher levels in the interaction of the pathogenic pv. *glycines* strain XP175 with cvs Clark or Clark 63, even though bacterial growth was not restricted in either cultivar.

Two to three additional antibacterial compounds of unknown identity also accumulated in certain interactions of pv. *glycines* with cvs Clark and Clark 63, but again, levels of these compounds appeared highest (based on the size of inhibitory zones obtained in the TLC bioassays) in the interaction of pathogenic pv. *glycines* strain XP175 with cvs Clark or Clark 63 where bacterial growth was not restricted.

From the results of the TLC bioassays of crude leaf extracts and the direct spotting bioassays which employed purified compounds, pathogenic strains of pv. *glycines* strains did not appear less susceptible to the inhibitory effects of glyceollin or of the unknown compounds with anti-bacterial activity.

Thus, we do not believe that accumulation of phytoalexins typical of the Leguminosae (isoflavonoid type) is involved in restriction of growth of nonpathogenic strains of pv. *glycines* or incompatible pv. *campestris*. The results of ultrastructural studies indicate that active immobilization of nonpathogenic strains of pv. *glycines* in the leaf intercellular spaces may be a resistance mechanism [10, 13] (Jones & Fett, unpublished results).

The involvement of phytoalexins in resistance of cv. Clark 63 to pathogenic strains of pv. *glycines* was not supported by the *in vivo* bacterial growth studies. We found no differences in growth of pathogenic pv. *glycines* strain XP175 in susceptible cv. Clark vs resistant cv. Clark 63 using bacterial suspensions containing 10^6 or 10^8 cfu ml⁻¹ for inoculation. Chamberlain [4] was the first to compare growth of a pathogenic strain of pv. *glycines* in a resistant and a susceptible soybean cultivar. He found pv. *glycines* was able to grow in the resistant cv. CNS but grew at a lower rate than in the susceptible cv. Lincoln. Chamberlain [4] also reported that no pustular lesions were formed on leaves of cv. CNS grown in the greenhouse when a range of bacterial concentrations were used for inoculation. In contrast, Groth & Brown [15] reported recently that resistance to pv. *glycines* was expressed in leaves of a resistant soybean cultivar by reduction in the number of pustular lesions but was not accompanied by a reduction in bacterial growth. Our results with cv. Clark 63 inoculated with pv. *glycines* strain XP175 agree with the latter findings.

A strong hypersensitive response similar to that which develops after inoculation of soybean leaves with certain incompatible phytopathogenic pseudomonads [25] occurred only when leaves of soybean cv. Clark were inoculated with high numbers (10^8 cfu ml⁻¹) of the nonpathogenic pv. *glycines* strain 1716. Inoculation of soybean leaves with similar levels of the other nonpathogenic strains of pv. *glycines* or an incompatible strain of pv. *campestris* did not lead to a strong hypersensitive reaction. However, this does not rule out a role for host cellular death in the restriction of bacterial growth seen in these reactions. Further studies at the light and electron microscope levels are required. Our finding that pv. *glycines* strain 1716 induced a strong HR without the accumulation of glyceollin supports previous findings [21] which indicated that in soybean the HR and glyceollin accumulation can occur independently.

Plant resistance to phytopathogens conditioned by recessive genes is unusual [24] and the type of resistance exhibited by cv. Clark 63 against pv. *glycines* also appears unusual. The presence of the *rpx* gene pair in cv. Clark 63 allows for rapid bacterial growth but suppresses symptom expression by reducing the number of pustular lesions formed accompanied by aborted growth and early necrosis of pustules that do form. Bacterial phytohormone production may be inhibited or an imbalance of the host's own phytohormones not allowed to occur to the extent as occurs in cv. Clark. The mechanism of resistance employed by cv. Clark 63 appears highly successful as no physiologic races of pv. *glycines* have appeared which can overcome resistance due to the *rpx* gene pair. We are continuing our studies on the nature of this resistance.

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