

Stress metabolite accumulation, bacterial growth and bacterial immobilization during host and nonhost responses of soybean to bacteria

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Hypersensitive response inducing (HR-inducing) strains of the heterologous (non-soybean) pathogens *Pseudomonas syringae* pv. *phaseolicola* and *P. syringae* pv. *syringae* attained populations in soybean leaves only three-fold to five-fold less than a compatible race of the soybean pathogen *P. syringae* pv. *glycinea*. Incompatible (HR-inducing) races of *P. syringae* pv. *glycinea* and a non-HR-inducing strain of the heterologous pathogen *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* showed more restricted growth. A non-HR-inducing strain of the heterologous pathogen *Erwinia carotovora* subsp. *atroseptica* and a strain of the saprophyte *Bacillus cereus* did not grow in soybean leaves. Incompatible races of *P. syringae* pv. *glycinea* and the heterologous pseudomonads induced accumulation of isoflavonoids (daidzein, formononetin, genistein, glyceollin) and isoflavone glucosides (daidzin, genistin, ononin). *Bacillus cereus* induced accumulation of isoflavone glucosides alone, and *C. flaccumfaciens* pv. *flaccumfaciens* and *E. carotovora* subsp. *atroseptica* did not induce accumulation of either type of stress metabolite. Of the purified compounds tested, glyceollin alone had significant antibacterial activity, but only against Gram-positive bacteria. By use of *in vitro* bioassays, no evidence was obtained to indicate that induction of additional inhibitory compounds or inhibitory activity due to total isoflavonoid content of inoculated leaf tissue was responsible for resistance.

Immobilization of bacteria by highly electron-dense material in intercellular spaces of soybean leaves occurred to the greatest extent with *E. carotovora* subsp. *atroseptica* and *B. cereus*, and to a lesser extent with *C. flaccumfaciens* pv. *flaccumfaciens*. No bacterial immobilization was evident at 48 h after inoculation with incompatible races of *P. syringae* pv. *glycinea* or heterologous pseudomonads.

INTRODUCTION

Bacterial induction of stress metabolites with antibacterial activity (phytoalexins) and bacterial cell immobilization at host mesophyll cell walls by electron-dense materials are two mechanisms of resistance which have been proposed to be responsible for host and nonhost (general) resistance of plants to phytopathogenic and saprophytic bacteria [24]. Much of the research on resistance mechanisms of soybean (*Glycine max* (L.) Merr.) towards phytopathogenic bacteria has focused on the role of the phytoalexin glyceollin (as used in this paper the term glyceollin refers to a mixture of glyceollin isomers [32]) in host resistance of soybean leaves to incompatible

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Abbreviations used in text: DMSO, dimethyl sulphoxide; HR, hypersensitive response; INT, *p*-iodonitrotetrazolium; KB, King's medium B agar; NDA, nutrient dextrose agar; TSB, violet tripticase soybroth.

hypersensitive response inducing (HR-inducing) races of the soybean pathogen *Pseudomonas syringae* pv. *glycinea*, causal agent of bacterial blight disease [29], and in nonhost resistance to heterologous (nonsoybean pathogens) phytopathogenic pseudomonads. It is well established that the soybean phytoalexin glyceollin accumulates faster and to higher levels in soybean leaves and cotyledons inoculated with incompatible races of *P. syringae* pv. *glycinea*, than with compatible races of this pathogen [3, 25, 36]. Heterologous pseudomonads can also induce accumulation of considerable amounts of glyceollin in inoculated soybean leaves [22, 25, 26]. Glyceollin has antibacterial activity [1, 11, 25] due to general disruption of membrane function [37]. In addition to glyceollin, numerous other isoflavonoids accumulate in soybean leaves inoculated with incompatible races of *P. syringae* pv. *glycinea* or heterologous pseudomonads [22, 25, 36]. It was reported that the isoflavone glucosides daidzin, genistin and ononin accumulate after inoculation of soybean leaves with *P. syringae* pv. *glycinea*, heterologous pseudomonads or xanthomonads [9, 36]. Evidence supporting an important role for glyceollin in resistance of soybean to incompatible races of *P. syringae* pv. *glycinea* has come from the work of Keen *et al.* In studies using a protein synthesis inhibitor [26], a temperature-sensitive strain of *P. syringae* pv. *glycinea* [20, 26] and from microscopic observation [19]. More recently, however, two reports [5, 18] have indicated that resistance mechanisms other than induced glyceollin accumulation may be important for resistance of soybean to pseudomonads. The importance of isoflavone glycoside accumulation in the resistance response of soybean to bacteria other than xanthomonads has not been critically assessed.

Indirect evidence that resistance of soybean leaves to incompatible races of *P. syringae* pv. *glycinea* and heterologous pseudomonads might be due to selective attachment and immobilization of bacterial cells comes from studies by Érsek *et al.* [6, 7]. They reported selective attachment of incompatible races of *P. syringae* pv. *glycinea* to isolated soybean leaf cells in culture [6]. An agglutinating factor was extracted from soybean leaves which preferentially agglutinated an incompatible race of *P. syringae* pv. *glycinea* and heterologous *Pseudomonas syringae* pv. *syringae* [7]. However, studies in this laboratory revealed no evidence for active bacterial immobilization of incompatible races of *P. syringae* pv. *glycinea* in soybean leaf intercellular spaces at 4 and 24 h after inoculation [10]. In that study, the interactions were not examined with the electron microscope after longer incubation periods.

While considerable research has been done on the role of phytoalexins in resistance of soybean to incompatible races of *P. syringae* pv. *glycinea* or heterologous pseudomonads, there has been a paucity of research on nonhost resistance of soybean to nonpseudomonad phytopathogenic bacteria. Weinstein *et al.* [38] reported that the primary antibacterial compound induced to accumulate in soybean cotyledons inoculated with *Erwinia carotovora* subsp. *carotovora* was not glyceollin but rather the structurally related pterocarpan glycinol (6a,3,9-trihydroxypterocarpan). Glycinol has not been reported to occur in uninoculated or inoculated soybean leaf tissue. Giddex *et al.* [14] found that *Erwinia amylovora* induced a HR in soybean primary leaves and that the intensity of the HR was dependent on postinoculation light intensities.

Recent studies in this laboratory indicated that accumulation of isoflavonoids and isoflavone glucosides is not responsible for resistance of soybean to *Xanthomonas*

campestris pv. *glycines*, causal agent of bacterial pustule disease of soybean, or to the heterologous pathogen *Xanthomonas campestris* pv. *campestris* [9].

This study attempted to determine whether isoflavonoid and isoflavone glucoside accumulation or bacterial immobilization were responsible for host resistance of soybean to incompatible races of *P. syringae* pv. *glycinea* and/or nonhost resistance to heterologous pseudomonads, nonpseudomonads and a saprophytic bacterium. A preliminary report has appeared [8].

MATERIALS AND METHODS

Bacterial Strains

The source plus host of origin (when known) of bacterial strains are listed in Table 1. *Pseudomonas syringae* pv. *phaseolicola* strains 1134 and 1137 were originally thought to belong to *P. syringae* pv. *glycinea* but this was recently shown to be incorrect [33].

TABLE 1.
Source and origin of bacterial strains

Bacterium	Strain	Source	Host of origin
<i>Bacillus cereus</i>	—	ERRC ^a	—
<i>B. licheniformis</i>	1264	NRRL ^b	—
<i>Corynebacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>	—	M. Schuster	<i>Phaseolus vulgaris</i>
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	SR8	A. Kelman	<i>Solanum tuberosum</i>
<i>E. carotovora</i> subsp. <i>carotovora</i>	E11	H. Moline	<i>Lactuca bremia</i>
<i>E. chrysanthemi</i>	SR239	A. Kelman	<i>Chrysanthemum leucanthemum</i>
<i>Escherichia coli</i> K12	23716	ATCC	—
<i>E. coli</i>	11775	ATCC	—
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>	A-29-2	W. Fett	<i>Glycine max</i>
	2159	NCPPB ^d	<i>Glycine max</i>
	J3-20-4A	W. Fett	<i>Glycine max</i>
	J3-17-2	W. Fett	<i>Glycine max</i>
<i>P. syringae</i> pv. <i>phaseolicola</i>	1134	NCPPB	<i>Neonotonia wighti</i> ^e
	1137	NCPPB	<i>Neonotonia wighti</i> ^e
<i>P. syringae</i> pv. <i>syringae</i>	Y30	D. J. Hagedorn	<i>P. vulgaris</i>
<i>Streptococcus lactis</i>	19435	ATCC	—

^aERRC = Eastern Regional Research Center, USDA, Philadelphia, PA, U.S.A.

^bNRRL = Northern Regional Research Center, USDA, Peoria, IL, U.S.A.

^cATCC = American Type Culture Collection, Rockville, MD, U.S.A.

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

^e*Neonotonia wighti* formerly classified as *Glycine javanica*.

Isoflavonoid standards

Glyceollin was isolated from soybean cv. Harosoy leaves inoculated with incompatible *P. syringae* pv. *glycinea* strain 2159 and purified as described by Fett [9]. Glyceollin isolated from soybean leaves is predominantly isomer III [11, 22]. A commercial sample of coumestrol (Eastman Kodak Co., NY, U.S.A.)† was further purified by

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

recrystallization from ethanol: water before use. Commercial samples of daidzein, formononetin and genistein (K & K Labs, ICN Pharmaceuticals, Inc., NY, U.S.A.) were used without further purification. Purified standards of daidzein, ononin and genistin were prepared as described by Osman & Fett [36]. A sample of glycinol was kindly provided by Dr R. Zacharius who had obtained it from Dr J. Ebel.

Plant propagation

Soybean cultivars Flambeau and Harosoy were propagated and maintained in a growth chamber (24 °C day, 20 °C night) as described previously [9].

Bacterial growth in vivo and accumulation of isoflavonoids and isoflavone glucosides

Growth of bacteria in leaves of soybean cvs Harosoy and Flambeau was followed simultaneously with accumulation of isoflavonoids and isoflavone glucosides. Inocula were prepared from 24 h old bacterial cultures grown either on King's medium B agar (KB) [28] (pseudomonads) or nutrient dextrose agar (NDA) (Difco nutrient broth plus 5 g l⁻¹ Difco yeast extract, 10 g l⁻¹ Difco dextrose, and 15 g l⁻¹ Bacto agar, Difco Laboratories, Detroit, MI, U.S.A.) (all others) at 24–28 °C. Bacterial cells of all strains (except for *Bacillus cereus*) were suspended in sterile water and the turbidity adjusted to OD_{600nm} = 0.10. Suspensions of *B. cereus* were adjusted to OD_{600nm} = 1.0. These turbidities corresponded to approximately 2–5 × 10⁷ colony-forming units (c.f.u.) ml⁻¹ as determined by standard dilution plating techniques.

Bacterial suspensions were forcibly sprayed against the abaxial side of unifoliate or trifoliate leaves until approximately 90% of the tissue appeared water-soaked. Inoculated leaves were rinsed well in running tap water; plants were set on the lab bench until all appearance of water-soaking disappeared (1–2 h) and then were returned to the growth chamber.

Growth of bacteria *in vivo* was followed by removal of leaf discs from inoculated areas, triturating in buffer, and determining the number of bacteria present by standard dilution plating techniques as described previously [9]. The remaining leaf material was extracted by boiling in 80% aq. EtOH for 10 min for determination of isoflavonoid and isoflavone glucoside levels. Extracts were then subjected to partitioning between diethyl ether:30% aq. EtOH (2:1), further separated by thin-layer chromatography (TLC) on 250 μm silica gel GF plates (Analtech, NJ, U.S.A.) and quantitated by high performance liquid chromatography (HPLC) on a normal phase 10 μm Porasil or a reverse-phase 10 μm Bondapak C₁₈ column (Waters Associates, MA, U.S.A.) [36]. All data presented in this paper are uncorrected for recovery efficiencies of approximately 60% determined by adding standards to uninoculated leaves before extracting in boiling 80% aq. EtOH [36]. For use in bioassays, leaf extracts from inoculated leaves were also obtained by a facilitated diffusion technique [23]. Analysis of extracts obtained by this method demonstrated the presence of isoflavonoids but not isoflavone glucosides.

Bioassays for presence of compounds with antifungal or antibacterial activity

The presence of antifungal compounds in diethyl ether or aq. EtOH soluble leaf

extracts was determined by a *Cladosporium* sp. TLC bioassay [27]. Leaf extracts (25 μ l, corresponding to 25 mg fresh weight of leaf tissue) in 95% EtOH were spotted onto silica gel GF, 250 μ m TLC plates along with appropriate purified standards (2 μ g each). Plates were developed either in cyclohexane:EtOAc (1:1, v/v) (for diethyl ether soluble extracts) or CHCl_3 :(CH_3)₂CO:methanol (20:6:5, v/v/v) for aq. EtOH soluble extracts). Purified standard compounds were also tested for their antifungal activity by spotting various levels onto TLC plates and performing the bioassay without plate development.

The presence of compounds with antibacterial activity in selected ethyl acetate soluble leaf extracts (extracted by the facilitated diffusion procedure) was also determined by a TLC bioassay. This bioassay utilized the abilities of *P. syringae* pv. *syringae* strain Y30 and *B. cereus* to hydrolyse aesculin giving a yellow coloured product [31] and *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* to reduce *p*-iodonitrotetrazolium violet (INT) (Sigma Chemical Co.) giving a violet-red coloured product [2]. Fifty to 100 μ l (50–100 mg fresh weight equivalents) of ethyl acetate soluble extracts in 95% EtOH were spotted onto 250 μ m silica gel GF TLC plates along with appropriate purified standard compounds, and plates were developed in cyclohexane:EtOAc (1:1, v/v). After plates had air-dried, bacterial suspensions ($\text{OD}_{600 \text{ nm}} = 1.0$) in trypticase soybroth (TSB) (BBL, Cockeysville, MD, U.S.A.) were lightly sprayed onto the plates. The wetted plates were incubated under 100% RH at room temperature overnight and then plates were sprayed either with a sterile aesculin-containing broth medium [31] or a sterile solution of INT at 4 mg ml⁻¹ water. Plates were kept under 100% RH for an additional 1–2 h, by which time a strong yellow background (in the case of aesculin hydrolysing bacteria) or a violet-red background (in the case of INT reducing bacteria) developed on the plates where metabolically active bacteria were present. The presence of compounds with antibacterial activity was indicated by a white area (due to the colour of silica gel) on the yellow or violet-red background. With each experiment, TLC plates which had not been spotted with leaf extracts were sprayed with sterile TSB alone, and 24 h later sprayed with either aesculin broth or INT as a control for microbial contamination. No yellow or violet-red colouration developed on the control plates, indicating an absence of contaminating micro-organisms with the ability for hydrolysing aesculin or reducing INT.

The TLC bioassays performed would not demonstrate antibacterial activity due to the presence of several isoflavonoids together as may occur *in vivo*. To determine if the total content of isoflavonoids in inoculated leaf tissue is inhibitory towards bacterial growth, bioassays involving the addition of ethyl acetate soluble leaf extracts to nutrient media were run. Up to 100 μ l (100 mg fresh weight equivalent) of ethyl acetate soluble leaf extract in methanol were added to sterilized culture vessels patterned after those described by Zalewski & Sequeira [40]. After the methanol evaporated, 4.4 ml of nutrient dextrose broth and 0.1 ml 95% EtOH (to give a final concentration of 2%) was added to each flask. Bacterial suspensions ($\text{OD}_{600 \text{ nm}} = 1.0$) in sterile water were prepared from 24 h bacterial cultures grown on NDA and 0.5 ml of bacterial suspension was added to each flask. Inoculated flasks without leaf extracts were included in each experiment. Duplicate flasks per treatment were used, and at least two separate experiments were carried out for each combination of leaf extract, concentration of leaf extract, and the bioassay bacterium. Flasks were shaken at 90

oscillations min^{-1} in a controlled temperature water bath at 26°C . Readings at $\text{OD}_{600\text{ nm}}$ were followed on a Bausch and Lomb Spectronic 88 colorimeter.

Purified standard compounds were tested for their antibacterial activity by use of two bioassays. The first was a TLC bioassay as described above, except that TLC plates were not developed. Purified isoflavonoids were tested at levels up to $80\ \mu\text{g}$ per spot and isoflavone glucosides at levels up to $124\ \mu\text{g}$ per spot. Antibacterial activity against *P. syringae* pv. *syringae* strain Y30, *C. flaccumfaciens* pv. *flaccumfaciens*, and *B. cereus* was tested with the TLC bioassay. These strains plus numerous other strains were also assayed using a direct spotting bioassay [39], as described previously [9]. All compounds were prepared at $1\text{--}2\ \text{mg ml}^{-1}$ in 95% EtOH or dimethyl-sulphoxide (DMSO) (Fisher Scientific Co., NJ, U.S.A.). Formononetin and ononin at $2\ \text{mg ml}^{-1}$ in 95% EtOH were gently heated immediately before use in order to insure complete solubility. For all test compounds $5\ \mu\text{l}$ volumes were spotted. Only formononetin at $2\ \text{mg ml}^{-1}$ in 95% EtOH or DMSO and coumestrol at $2\ \text{mg ml}^{-1}$ in DMSO left a visible precipitate on the agar surface after disappearance of solvent. Streptomycin sulphate (Böhringer Mannheim, West Germany) at $2\ \text{mg ml}^{-1}$ in water was used ($2\ \mu\text{l}$ volumes) as a positive control. Assay plates were incubated for 48 h at 28°C .

Electron microscopy

Bacterial cells from cultures grown overnight at 28°C on KB (pseudomonads) or NDA (all others) were suspended in sterile water and washed three times by repeated centrifugation. Washed cells of all strains except *B. cereus* were suspended in sterile water and turbidity adjusted to $\text{OD}_{600\text{ nm}} = 1.5$ (approx. 7×10^8 to 3×10^9 c.f.u. ml^{-1}). Washed cells of *B. cereus* were suspended in sterile water and turbidity adjusted to $\text{OD}_{600\text{ nm}} = 2.0$ (approx. 4×10^7 c.f.u. ml^{-1}). Unifoliate or first trifoliate leaves of cv. Harosoy were inoculated by spraying as described above. Inoculated plants were returned to the growth chamber (24°C day, 20°C night) after all appearances of water-soaking disappeared (approximately 2 h). Leaf tissue from the inoculated areas was excised 48 h after inoculation, prepared for electron microscopy and examined as described by Fett & Jones [10].

RESULTS

Bacterial growth in vivo and symptomology

Of the pseudomonad strains tested for growth in leaves of cv. Harosoy compatible *P. syringae* pv. *glycinea* strain A-29-2 (race 4) [12] reached the highest populations (approximately 1×10^9 c.f.u. cm^{-2} leaf area) within 4 days after inoculation [Fig. 1(a)]. This bacterial strain induced the formation of water-soaked lesions typical of bacterial blight disease of soybean [29], first visible to the naked eye within 2 days of inoculation. Growth of incompatible *P. syringae* pv. *glycinea* strain 2159 (race 1) [12] was the most restricted of all pseudomonads tested with populations reaching only approximately 2×10^7 c.f.u. cm^{-2} leaf area [Fig. 1(a)]. This strain induced a HR moderate in intensity characterized by a "silvering" of the abaxial leaf surface at 24 h followed by browning [25] first evident by 2 days after inoculation. The two heterologous pseudomonads tested exhibited more prolific growth with final populations reaching intermediate values between the two *P. syringae* pv. *glycinea*

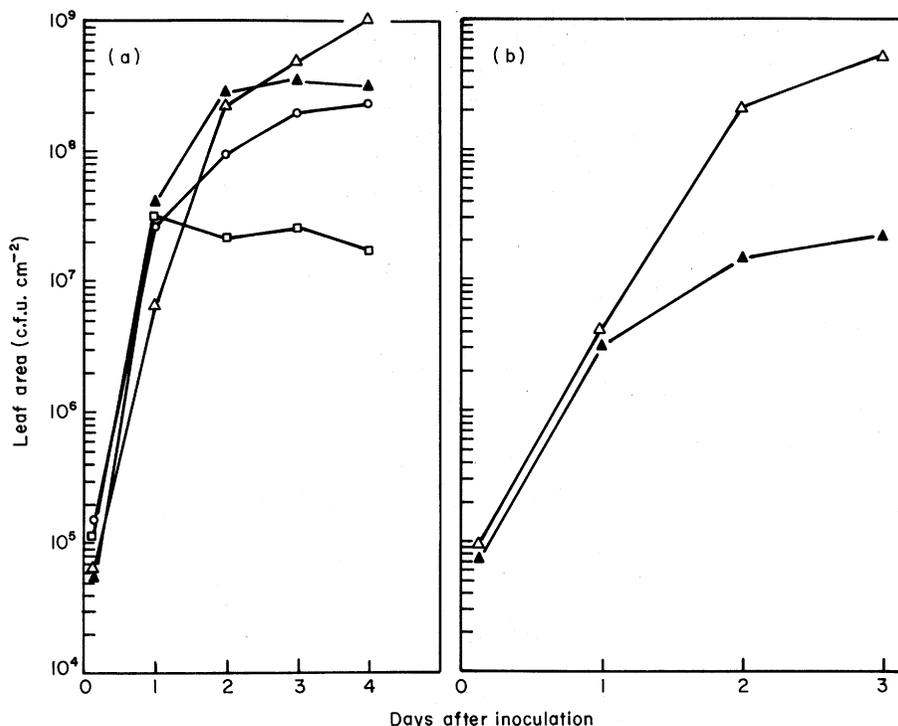


Fig. 1. Bacterial growth in leaves of soybean after inoculation with bacterial suspensions containing approx. $2-5 \times 10^7$ c.f.u. ml⁻¹. (a) cv. Harosoy. Δ , *Pseudomonas syringae* pv. *glycinea* strain A-29-2; \square , *P. syringae* pv. *glycinea* strain 2159; \circ , *P. syringae* pv. *phaseolicola* strain 1134; \blacktriangle , *P. syringae* pv. *syringae* strain Y30. (b) cv. Flambeau. Δ , *P. syringae* pv. *glycinea* strain J3-20-4A; \blacktriangle , *P. syringae* pv. *glycinea* strain J3-17-2.

strains. *Pseudomonas syringae* pv. *syringae* strain Y30 (a pathogen of *Phaseolus vulgaris*) grew at a rate comparable with *P. syringae* pv. *glycinea* strain A-29-2 for the first 48 h after inoculation and attained populations (3×10^8 c.f.u. cm⁻² leaf area) only three-fold lower than the compatible *P. syringae* pv. *glycinea* strain A-29-2 by 3-4 days after inoculation [Fig. 1(a)]. *Pseudomonas syringae* pv. *syringae* strain Y30 induced a very strong HR with much tissue necrosis evident by 2 days after inoculation. *Pseudomonas syringae* pv. *phaseolicola* strain 1134 (also a pathogen of *P. vulgaris*) attained populations (2×10^8 c.f.u. cm⁻² leaf area) only five-fold lower than the compatible strain of *P. syringae* pv. *glycinea* [Fig. 1(a)]. *Pseudomonas syringae* pv. *phaseolicola* strain 1134 induced a HR similar in intensity to the incompatible *P. syringae* pv. *glycinea* strain 2159.

In leaves of soybean cv. Flambeau, bacterial populations were followed only up to 3 days after inoculation. In this time period, compatible *P. syringae* pv. *glycinea* strain J3-20-4A (race 4) [12] reached populations of approximately 5×10^8 c.f.u. cm⁻² leaf area [Fig. 1(b)], while incompatible *P. syringae* pv. *glycinea* strain J3-17-2 (race 5) [12] reached approximately 2×10^7 c.f.u. cm⁻² leaf area. By 2 days after inoculation compatible *P. syringae* pv. *glycinea* strain J3-20-4A induced the formation of water-

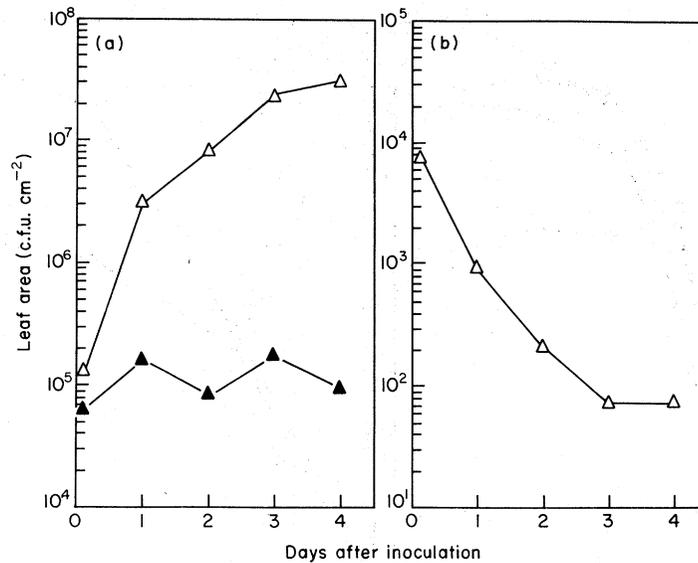


FIG. 2. Bacterial growth in leaves of soybean cv. Harosoy after inoculation with bacterial suspensions containing approx. $2-5 \times 10^7$ c.f.u. ml⁻¹. (a) Δ , *Corynebacterium flaccumfaciens* pv. *flaccumfaciens*; \blacktriangle , *Erwinia carotovora* subsp. *atroseptica* strain SR8. (b) Δ , *Bacillus cereus*.

soaked lesions and incompatible *P. syringae* pv. *glycinea* strain J3-17-2 induced a HR moderate in intensity.

Two heterologous nonpseudomonad bacterial strains were tested for growth and symptom production on leaves of cv. Harosoy. Populations of *C. flaccumfaciens* pv. *flaccumfaciens* (a pathogen of *P. vulgaris*) reached a maximum of approximately 3×10^8 c.f.u. cm⁻² leaf area by 4 days after inoculation [Fig. 2(a)]. Populations of *Erwinia carotovora* subsp. *atroseptica* (a pathogen of potato) strain SR8 did not increase or decrease over the experimental period [Fig. 2(a)]. Neither bacteria induced a HR, but rather induced a light green chlorosis of the incubated tissue first evident by 2 days after inoculation.

The saprophyte *B. cereus* showed a 100-fold drop in population by 3 days after inoculation of leaves of cv. Harosoy [Fig. 2(b)]. No symptoms were evident in this interaction over the experimental period.

Accumulation of isoflavonoids and isoflavone glucosides

All HR-inducing pseudomonads tested induced more rapid and higher accumulation of isoflavonoids than did compatible strains of *P. syringae* pv. *glycinea* in leaves of cvs Harosoy and Flambeau. The levels of isoflavonoids found 3 days after inoculation are shown in Table 2.

In leaves of cv. Harosoy, the HR-inducing pseudomonads (*P. syringae* pv. *glycinea* strain 2159, *P. syringae* pv. *phaseolicola* strain 1134, and *P. syringae* pv. *syringae* strain Y30), but not the compatible *P. syringae* pv. *glycinea* strain A-29-2, induced accumulation of isoflavone glucosides with daidzin accumulating to the highest levels

TABLE 2

Accumulation of isoflavonoids and isoflavone glucosides in soybean leaves ($\mu\text{g g}^{-1}$ f. wt) 3 days after inoculation with bacteria

Cultivar-bacterium interaction	Bacterial strain	Symptoms ^a	$\mu\text{g g}^{-1}$ fresh weight																	
			Glyceollin		Coumestrol		Daidzein		Formononetin		Genistein		Glycinol		Daidzin		Ononin		Genistin	
			Exp.		Exp.		Exp.		Exp.		Exp.		Exp.		Exp.		Exp.		Exp.	
Harosoy																				
<i>P. syringae</i> pv. <i>glycinea</i>	A-29-2	WS	— ^b	—	2 ^c	—	TR ^d	—	1	—	—	—	ND ^e	4	TR	—	—	—	3	2
	2159	HR	83	235	37	393	1	37	48	35	TR	2	—	61	95	8	11	19	8	
pv. <i>phaseolicola</i>	1134	HR	21	63	3	44	—	29	3	17	TR	17	ND	33	107	5	65	17	16	
pv. <i>syringae</i>	Y30	HR	55	100	128	96	45	219	69	280	ND	1	ND	56	158	11	37	14	25	
<i>C. flaccumfaciens</i> pv. <i>flaccumfaciens</i>	C	C	TR	—	ND	ND	ND	ND	ND	ND	ND	ND	—	TR	—	—	TR	TR	TR	
<i>E. carotovora</i> subsp. <i>atroseptica</i>	SR8	C	TR	—	ND	ND	ND	ND	ND	ND	ND	ND	—	TR	TR	—	—	TR	—	
<i>B. cereus</i>	NS	NS	—	—	4	—	1	—	1	—	ND	—	ND	10	7	10	10	20	34	
Sterile water	NS	NS	—	—	—	—	—	—	—	TR	—	—	—	3	1	2	—	—	6	
Flambeau																				
<i>P. syringae</i> pv. <i>glycinea</i>	J3-20-4A	WS	TR	TR	—	—	—	—	—	—	—	—	ND	38	18	1	1	18	9	
	J3-17-2	HR	161	729	108	147	25	43	24	14	2	10	ND	52	23	1	1	15	4	
Sterile water	NS	NS	—	—	—	—	—	—	—	—	—	—	ND	—	TR	—	—	—	2	

^aWS, water-soaked lesions; HR, hypersensitive response; C, slight chlorosis; NS, no symptoms.^b—, None detected.^cValues are uncorrected for extraction efficiencies.^dTR, trace ($<1.0 \mu\text{g g}^{-1}$ f. wt).^eND, not determined.

(Table 2). In leaves of cv. Flambeau, compatible *P. syringae* pv. *glycinea* strain J3-20-4A and incompatible *P. syringae* pv. *glycinea* strain J3-17-2 induced the accumulation of similar levels of daidzin and genestin. Ononin did not accumulate in either interaction.

The nonpseudomonad heterologous phytopathogens *C. flaccumfaciens* pv. *flaccumfaciens* and *E. carotovora* subsp. *atroseptica* did not induce the accumulation of glyceollin or glycinol (the only isoflavonoids quantitated for these two bacteria) and the saprophyte *B. cereus* did not induce the accumulation of any of the isoflavonoids in leaves of cv. Harosoy (Table 2).

Corynebacterium flaccumfaciens pv. *flaccumfaciens* and *E. carotovora* subsp. *atroseptica* strain SR8 did not induce accumulation of isoflavone glucosides, while *B. cereus* induced low levels of these compounds to accumulate in leaves of cv. Harosoy (Table 2).

TLC of crude diethyl ether soluble leaf extracts indicated that several blue-fluorescent and yellow-fluorescent compounds (visible when the plates were examined under 350 nm light) of unknown identity accumulated during the interaction of incompatible strains of *P. syringae* pv. *glycinea* or heterologous pseudomonads with leaves of cvs. Harosoy or Flambeau (Fig. 3). Accumulation of the fluorescent compounds was variable, and not all of the compounds accumulated in each experiment.

Antifungal and antibacterial activity of crude leaf extracts and purified compounds

Diethyl ether soluble extracts prepared from leaves of cvs Harosoy and Flambeau inoculated with bacteria or sprayed with sterile water alone were tested for the presence of compounds with antifungal activity using a *Cladosporium* sp. TLC bioassay

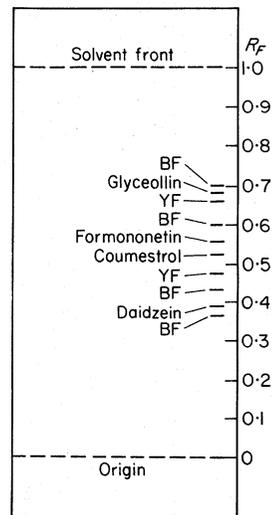


FIG. 3. Location of blue-fluorescent (BF) and yellow-fluorescent (YF) (visible under 350 nm light) stress metabolites when diethyl ether soluble leaf extracts were chromatographed on 250 μ m Silica gel GF TLC plates using cyclohexane:EtOAc (1:1) for development.

[27]. Only the diethyl ether extracts obtained from the interactions of incompatible *P. syringae* pv. *glycinea* strain 2159, *P. syringae* pv. *phaseolicola* strain 1134 and *P. syringae* pv. *syringae* strain Y30 with cv. Harosoy and incompatible *P. syringae* pv. *glycinea* strain J3-17-2 with cv. Flambeau exhibited inhibitory activity. All of these interactions led to the presence of inhibitory compounds in the diethyl ether leaf extracts by 1–2 days after inoculation. The two main areas of inhibition were centred at $R_F = 0.67$ and 0.55 . A smaller area of inhibition centred at $R_F = 0.39$ was occasionally present for the interaction of *P. syringae* pv. *glycinea* strain 2159 with cv. Harosoy. These R_F s were similar to those found for standards of glyceollin (0.67), genistein (0.59), formononetin (0.55), coumestrol (0.52), glycinol (0.44) and daidzein (0.39). Glyceollin is well known to have inhibitory activity against *Cladosporium* [11, 27] and was active at 2 μg in our bioassays. Purified standards of daidzein, coumestrol, formononetin, genistein and glycinol (assayed up to 200, 50, 200, 100 and 40 μg , respectively) were not inhibitory towards the *Cladosporium* sp. when tested using the TLC bioassay.

Aqueous EtOH soluble extracts prepared of leaves from cv. Harosoy inoculated with *P. syringae* pv. *glycinea* strain 2159, *P. syringae* pv. *phaseolicola* strain 1134 or sterile water alone had no inhibitory activity in this bioassay.

Leaf extracts were examined for the presence of compounds with antibacterial activity by two methods. In the first method, ethyl acetate soluble leaf extracts (obtained by the facilitated diffusion procedure) were screened for antibacterial activity by a TLC bioassay utilizing the abilities of *P. syringae* pv. *syringae* strain Y30 and *B. cereus* to hydrolyse aesculin and of *C. flaccumfaciens* pv. *flaccumfaciens* to reduce the tetrazolium salt INT. Strains of *P. syringae* pv. *glycinea* could not be used as the bioassay organism since they do not hydrolyse aesculin [36]. In addition, strains of *P. syringae* pv. *glycinea* did not give reproducible reduction of any of several tetrazolium salts tested including INT. No antibacterial activity towards *P. syringae* pv. *syringae* strain Y30 was found in extracts (50–100 mg f. wt equivalents) prepared from leaves of cv. Harosoy showing a HR due to inoculation with *P. syringae* pv. *glycinea* strain 2159 or *P. syringae* pv. *phaseolicola* strain 1134.

Ethyl acetate soluble extracts from leaves of cv. Harosoy inoculated with *P. syringae* pv. *phaseolicola* strain 1134 did contain at least four compounds inhibitory towards *C. flaccumfaciens* pv. *flaccumfaciens* with areas of inhibition centred at R_F 0.67, 0.53, 0.42 and 0.36. Similar extracts from leaves of cv. Harosoy inoculated with *P. syringae* pv. *glycinea* strain 2159 contained at least five compounds with inhibitory activity towards *C. flaccumfaciens* pv. *flaccumfaciens* with areas of inhibition centred at $R_F = 0.67$, 0.57, 0.52, 0.39 and 0.36. The same extracts tested against *B. cereus* consistently gave an inhibitory area at $R_F = 0.67$ and occasionally one at $R_F = 0.52$. Comparable extracts from cv. Harosoy leaves sprayed with sterile water alone produced no areas of inhibition in this bioassay.

The areas of bacterial inhibition centred at R_F 0.67, 0.57, 0.53, 0.52, 0.42 and 0.39 were at similar R_F to glyceollin (0.67), genistein (0.59), formononetin (0.55), coumestrol (0.52), glycinol (0.44) and daidzein (0.39). However, when purified standards of these compounds (tested up to 80 μg per spot), plus daidzin, genistin and ononin (tested up to 124 μg per spot) were assayed against *C. flaccumfaciens* pv. *flaccumfaciens* and *B. cereus*, only glyceollin was strongly inhibitory giving inhibition at ≥ 7.5 and 1.5 μg per spot, respectively. Coumestrol at ≥ 2 μg appeared slightly

TABLE 3.

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

Bacterium	Strain	Glyceollin (10 µg in EtOH)	Glycinol (10 µg in EtOH)	Coumestrol (10 µg in DMSO)	Daidzein		Daidzin (10 µg in EtOH)	Formononetin		Ononin (10 µg in EtOH)	Genistein (10 µg in EtOH)	Genistin (10 µg in EtOH)
					(5 µg in EtOH)	(10 µg in DMSO)		(10 µg in EtOH)	(10 µg in DMSO)			
Gram-positive												
<i>Bacillus cereus</i>	—	2·3 ^a	0	ND ^b	1·6	3·0	0	0	0	0	1·1	0
<i>B. licheniformis</i>	1264	(3·0) ^c	ND	(4·0)	0	ND	0	0	ND	0·7	0·8	0
<i>Corynebacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>	—	1·8	0	(0)	0	0	0	0	0	0	0·4	0
<i>Streptococcus lactis</i>	19435	(4·0)	ND	ND	0	ND	0	0	0	1·0	0	0
Gram-negative												
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	SR8	0	0	0·3	0	1·3	0	0	0	ND	ND	ND
<i>E. carotovora</i> subsp. <i>carotovora</i>	E11	0	ND	0	0	0·3	ND	0	0	ND	ND	ND
<i>E. chrysanthemi</i>	239	0	ND	0	0	0	ND	0	0	ND	0	ND
<i>Escherichia coli</i> K12	23716	0	ND	0	0	0	ND	0	0	ND	0	ND
<i>E. coli</i>	11775	0	ND	0	0	0	ND	0	0	ND	0	ND
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>	2159	(0)	0	(0)	0	0	0	0	0	0	0	0
	A-29-2	(0)	0	(0)	1·3	0	0	0	0	0	0	0·8
	J3-20-4A	ND	ND	ND	0	0·5	0	ND	0	0	0	0
	J3-17-2	(0)	ND	(0)	0	0	0	ND	0	0	0	0
<i>P. syringae</i> pv. <i>phaseolicola</i>	1134	(0)	ND	(0)	0	0	0	ND	0	0	0	0
	1137	ND	ND	ND	0	0	0	ND	0	0	0	0
<i>P. syringae</i> pv. <i>syringae</i>	Y30	0	ND	ND	0	0	0	0	0	0	0	0

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

^bND, not determined.

^cValues in parentheses taken from Fett & Osman [11].

inhibitory towards *C. flaccumfaciens* pv. *flaccumfaciens*. Coumestrol was not tested for inhibition of *B. cereus* using this bioassay system. None of the compounds inhibited *P. syringae* pv. *syringae* strain Y30. Due to the limited amount of glycinol available glycinol was not tested in these bioassays.

The antibacterial activity of purified isoflavonoids and isoflavone glucosides was also determined by a direct spotting bioassay (Table 3). It was determined previously that this assay is more sensitive than the traditional impregnated filter disc and agar incorporation bioassays [11]. None of the bacterial strains listed in Table 3 were more than slightly inhibited (inhibition rating of ≤ 1) by 95% EtOH or DMSO alone. In an earlier study which made use of the direct spotting assay [11], it was found that 95% EtOH alone was highly inhibitory towards the same strain of *C. flaccumfaciens* pv. *flaccumfaciens* used in the present study. Different commercial preparations or different lots of 95% EtOH from the same manufacturer appear to contain inhibitory substances in variable amounts. Values reported in Table 2 have been corrected for control values. None of the isoflavonoids or isoflavone glucosides had more than slight inhibitory activity against the Gram-negative bacteria assayed (Table 3). Against the Gram-positive bacteria, glyceollin was inhibitory towards all strains and coumestrol was highly inhibitory towards *Bacillus licheniformis* strain 1264. Streptomycin sulphate at 2 μg gave complete inhibition (rating of 4.0) of growth of all bacterial strains (data not shown).

To determine if the total admixture of isoflavonoids found in extracts from inoculated leaves had distinctive inhibitory capabilities, we carried out bioassays in a system containing nutrient dextrose broth (prepared as for NDA but with agar omitted), leaf extracts, 2% EtOH added to aid solubilization, and one strain of test bacterium. All leaf extracts used in these assays were obtained by the facilitated diffusion procedure from leaves of cv. Harosoy 3 days after inoculation with bacteria. Leaf extracts were tested for inhibitory activity at levels of 5–100 μl (5–100 mg f. wt equivalents).

Extracts from leaves showing a HR due to inoculation with either *P. syringae* pv. *glycinea* strain 2159 or *P. syringae* pv. *phaseolicola* strain 1134 increased lag times up to three-fold and generation times up to two-fold for the Gram-negative test bacteria *P. syringae* pv. *glycinea* strains A-29-2 or 2159, *P. syringae* pv. *phaseolicola* strain 1134, and *P. syringae* pv. *syringae* strain Y30. However, extracts prepared from leaves sprayed with sterile water alone were at least as inhibitory as extracts from the leaves inoculated with bacteria. The same results were obtained when extracts of leaves inoculated with *E. carotovora* subsp. *atroseptica* strain SR8 were tested for inhibitory activity against this same strain. The extracts (100 μl) from leaves sprayed with sterile water alone produced the greatest inhibition giving a two- to four-fold increase in lag time and an increase in generation time from 1.9 to 2.6 h.

Leaf extracts from inoculated leaves as well as water-sprayed control leaves were more inhibitory towards the Gram-positive than the Gram-negative test bacteria. For instance, 100 μl of extract from leaves inoculated with *P. syringae* pv. *glycinea* strain 2159 completely inhibited growth of *B. cereus* and this same level of extract from leaves inoculated with *C. flaccumfaciens* pv. *flaccumfaciens* completely inhibited growth of *C. flaccumfaciens* pv. *flaccumfaciens*. Comparable levels of extracts from water-sprayed control leaves were once again equally as or more inhibitory towards bacterial growth

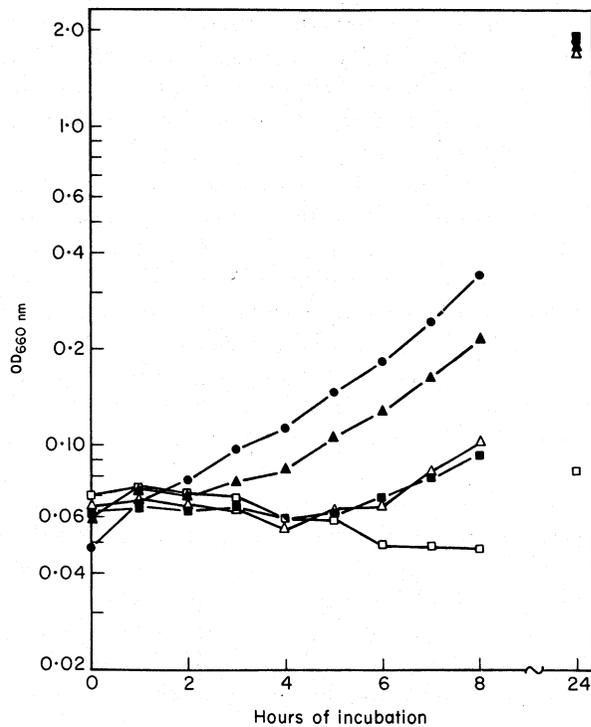


FIG. 4. Effect of addition of extracts prepared from leaves of soybean cv. Harosoy 3 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola* strain 1134 on growth of *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* in nutrient dextrose broth. No added leaf extract (●); 50 (■) or 100 (□) mg f. wt equivalents of extracts from leaves inoculated with bacteria; 50 (▲) or 100 (△) mg f. wt equivalents of extracts from leaves sprayed with sterile water alone.

Ultrastructural observations

High numbers of bacteria were present in leaf intercellular spaces of cv. Harosoy 48 h after inoculation with compatible *P. syringae* pv. *glycinea* strains A-29-2 or with incompatible HR-inducing *P. syringae* pv. *glycinea* strain 2159, *P. syringae* pv. *phaseolicola* strain 1134, and *P. syringae* pv. *syringae* strain Y30 cells [Fig. 5(a)]. There was much host cell collapse but no evidence of immobilization of bacteria by electron-dense material in any of these interactions. In contrast, there was immobilization of *E. carotovora* subsp. *atroseptica* strain SR8 in intercellular leaf spaces. The majority of cells of *E. carotovora* subsp. *atroseptica* strain SR8 were covered by highly electron-dense material and many of the immobilized bacterial cells had condensed cytoplasm [Fig. 5(b)]. No host cell collapse was seen. The majority of cells of *C. flaccumfaciens* pv. *flaccumfaciens* were free in the intercellular spaces, but occasionally bacterial cells were

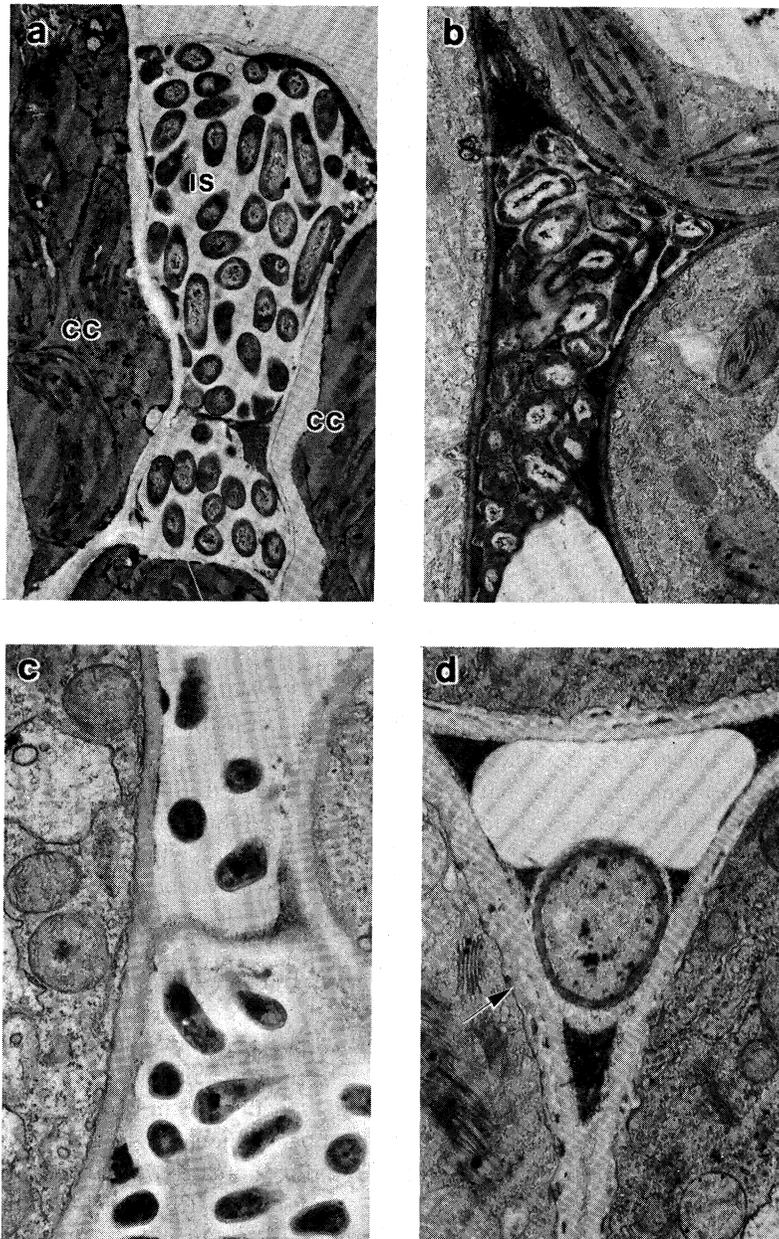


FIG. 5. Transmission electron micrographs of hypersensitive response (HR) inducing (*Pseudomonas syringae* pv. *glycinea* strain 2159) or non-HR-inducing (*Erwinia carotovora* subsp. *atroseptica* strain SR8, *Corynebacterium flaccumfaciens* pv. *flaccumfaciens*, and *Bacillus cereus*) bacteria in leaves of cv. Harosoy 48 h after inoculation. (a) *Pseudomonas syringae* pv. *glycinea* strain 2159 free in the intercellular space (IS) between collapsed mesophyll cells (cc). $\times 8000$. (b) *Erwinia carotovora* subsp. *atroseptica* SR8 immobilized by heavy-staining material at junction of mesophyll cells. $\times 8000$. (c) *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* observed in this part of the leaf space both free and surrounded by fibrils. $\times 16\ 000$. (d) *Bacillus cereus* partially covered by fibrillar material and heavy-staining material at a junction of mesophyll cells. Additional material has accumulated in the inside of the mesophyll cell wall (arrow). $\times 16\ 000$.

surrounded by fibrillar [Fig. 5(c)] or more highly electron-dense material (not shown). Again, no host cell collapse was noted.

Only a few cells of the saprophyte *B. cereus* were seen under the electron microscope. Most cells of *B. cereus* were embedded in electron-dense material usually at junctions of host cells [Fig. 5(d)]. In some instances the plasmalemma of host cells in close proximity to cells of *B. cereus* was pulled away from the host cell wall and structured material accumulated in the space between the host cell wall and plasmalemma [arrow in Fig. 5(d)]. No host cell collapse was seen.

DISCUSSION

Compatible phytopathogenic bacteria are usually reported to attain 200-fold to 10 000-fold higher populations in plant leaves than do HR-inducing heterologous bacteria [4, 9, 21, 35]. Compatible strains of *P. syringae* pv. *glycinea* have been reported to attain approximately 1000-fold higher populations in soybean leaves than do HR-inducing incompatible strains of this bacterium [5, 26] when bacterial inocula contained 10^6 – 10^8 c.f.u. ml⁻¹. In our studies, compatible strains of *P. syringae* pv. *glycinea* attained populations only 25-fold to 50-fold higher than incompatible strains of *P. syringae* pv. *glycinea* in leaves of soybean cvs. Harosoy and Flambeau and only three-fold to five-fold higher than HR-inducing strains of heterologous pathogens *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *syringae*. Ercolani & Crosse [4] reported a similar five-fold reduction in final populations of a heterologous strain of *P. syringae* pv. *phaseolicola* in leaves of cherry when compared with a homologous strain of *Pseudomonas syringae* pv. *morsprunorum* when bacterial inocula contained 10^7 c.f.u. ml⁻¹. In both soybean and cherry leaves, such high populations of heterologous bacteria were attained without visible water-soaking of the affected tissues, which suggests that very high concentrations of bacteria in leaf intercellular spaces can be attained without the degree of induced water loss from leaf cells which would lead to visible water-soaking of the leaf tissues.

That a true HR did occur in soybean leaves by 48 h after inoculation with *P. syringae* pv. *glycinea* strain 2159 was supported by ultrastructural observations [Fig. 5(a)]. Similar results were found for *P. syringae* pv. *phaseolicola* strain 1134 and *P. syringae* pv. *syringae* strain Y30 (data not shown). The HR in soybean to phytopathogenic bacteria appears to be much less effective in restricting bacterial growth than the HR in tobacco leaves [34]. In tobacco inoculated with incompatible bacteria host cell membrane permeability rapidly increases 7–9 h after bacterial infiltration followed by extensive host cell collapse at 8–11 h [30]. By 24 h, the infiltrated tobacco leaf tissue is totally desiccated with a tan papery appearance. In contrast, with soybean little increase in host cell permeability occurs by 3 days after inoculation [25] while tissue necrosis as indicated by tissue browning and collapse is not evident until 48 h after inoculation [25]. Our own visual observations would indicate that the degree of tissue desiccation in soybean at 48 h after inoculation is less than in tobacco by 24 h after inoculation.

Consistent with earlier reports [5, 25], we found HR-inducing strains of *P. syringae* pv. *glycinea* and heterologous pseudomonads to induce more rapid and higher accumulation of isoflavonoids than compatible strains of *P. syringae* pv. *glycinea* in

soybean leaves. The levels of glyceollin isomers ($21\text{--}729\ \mu\text{g g}^{-1}$ f. wt) which we found to accumulate in soybean leaves after inoculation with incompatible pseudomonads were, in general, lower than those reported by others ($285\text{--}1000\ \mu\text{g g}^{-1}$ f. wt) for soybean inoculated with incompatible races of *P. syringae* pv. *glycinea*. Glycinol, which has been reported to be the major phytoalexin accumulating in soybean cotyledons inoculated with *E. carotovora* subsp. *carotovora* [38] and has bacteriostatic activity against a variety of bacteria including *E. carotovora* subsp. *carotovora*, *Bacillus subtilis* and *P. syringae* pv. *glycinea* [37, 38], did not accumulate in soybean leaves inoculated with incompatible *P. syringae* pv. *glycinea* strain 2159. Ingham *et al.* [22] also failed to detect accumulation of glycinol in soybean leaves inoculated with the incompatible pathogen *Pseudomonas syringae* pv. *lisi*. None of the purified isoflavonoids when tested alone by direct spotting or TLC bioassays were strongly inhibitory towards the pseudomonads. Utilizing bioassays run in liquid media, glyceollin was previously found to inhibit *P. syringae* pv. *glycinea* strains 2159 and A-29-2 and *P. syringae* pv. *phaseolicola* strain 1134 confirming earlier reports demonstrating the antibacterial nature of glyceollin [1, 25, 37]. While *P. syringae* pv. *glycinea* strain A-29-2 had a minimal inhibitory concentration of $12.5\ \mu\text{g ml}^{-1}$ for glyceollin, strain 2159 was very resistant to glyceollin with a minimum inhibitory concentration of $400\ \mu\text{g ml}^{-1}$ [11]. The accumulation of additional isoflavonoids with antibacterial activity against pseudomonads was not demonstrated by TLC bioassays.

Two recent reports indicate that accumulation of glyceollin may not be responsible for resistance of soybean leaves to pseudomonads. Érsek & Hevesi [5] determined that a strain of *P. syringae* pv. *syringae* had growth kinetics in leaves of soybean cv. Merit similar to those of HR-inducing strains of *P. syringae* pv. *glycinea*, but that the *P. syringae* pv. *syringae* strain did not induce either glyceollin accumulation or an HR. They concluded that these two host responses may not be responsible for resistance of soybean to pseudomonads. Holliday & Keen [18] found that a post-inoculation treatment of soybean with the herbicide glyphosate severely reduced the level of glyceollin which accumulated after inoculation of leaves with an incompatible race of *P. syringae* pv. *glycinea* but not the HR. Inhibition of glyceollin accumulation led to an increase of bacterial growth, but bacterial populations attained were still eight-fold less than when a compatible race of *P. syringae* pv. *glycinea* was used for inoculation. They concluded that glyceollin accumulation was responsible for some restriction of bacterial growth but that other resistance mechanisms such as the HR were also functioning. Only glyceollin levels were quantitated in their study, but glyphosate is an inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase [17], an enzyme in the shikimate pathway to aromatic amino acid synthesis including phenylalanine. Thus, glyphosate treatment would be expected to also inhibit *de novo* synthesis of coumestrol, daidzein and formononetin, and all other isoflavonoids. The present *in vitro* bioassays with crude leaf extracts also indicated that the total isoflavonoid content of leaves of soybean showing an HR is not responsible for inhibition of bacterial growth *in vivo*.

In leaves of cv. Harosoy, isoflavone glucosides accumulated more rapidly and to higher levels after inoculation with HR-inducing pseudomonads than with a compatible strain of *P. syringae* pv. *glycinea*. This was not found for leaves of cv. Flambeau where similar levels of isoflavone glucosides accumulated after inoculation

with compatible or HR-inducing incompatible strains of *P. syringae* pv. *glycinea*. The latter finding, plus the inactivity of the isoflavone glucosides against pseudomonads in *in vitro* bioassays, rule out a role for these stress metabolites in resistance to HR-inducing pseudomonads.

Accumulation of isoflavonoids and isoflavone glucosides or a HR were not responsible for resistance of leaves of cv. Harosoy to the heterologous pathogens *E. carotovora* subsp. *atroseptica*, *C. flaccumfaciens* pv. *flaccumfaciens*, and the saprophyte *B. cereus*. *Erwinia carotovora* subsp. *atroseptica* did not induce accumulation of isoflavonoids or isoflavone glucosides and extracts from leaves inoculated with *E. carotovora* subsp. *atroseptica* were no more inhibitory towards growth of this strain than comparable control extracts. Purified standards were not inhibitory towards this bacterium in *in vitro* bioassays. *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* and *B. cereus* were inhibited by glyceollin and glyceollin plus daidzein, respectively in *in vitro* bioassays, but neither of these Gram-positive bacteria induced accumulation of isoflavonoids *in vivo*. *Bacillus cereus* did induce accumulation of low levels of isoflavone glucosides but these compounds were not inhibitory to *B. cereus* in *in vitro* bioassays.

The greater sensitivity of Gram-positive bacteria over that of Gram-negative bacteria to glyceollin and to certain other isoflavonoid-type compounds were indicated by results of direct spotting and TLC bioassays and confirms the findings of others [11, 15, 16].

Bacterial immobilization at leaf mesophyll cell walls is not responsible for resistance of soybean to HR-inducing pseudomonads. In a previous study [10], no evidence was found for immobilization of bacterial cells at the leaf mesophyll cell walls as an active defense response of leaves of cvs. Harosoy and Flambeau to incompatible HR-inducing races of *P. syringae* pv. *glycinea* by 24 h after inoculation. In the present study the ultrastructural observations were extended until 48 h after inoculation, a time when growth *in vivo* of incompatible races of *P. syringae* pv. *glycinea* is restricted. Again, there was no evidence for bacterial immobilization as a defense response. Results were similar for heterologous pseudomonads with no evidence for bacterial immobilization 48 h after inoculation.

The strongest evidence for a role of bacterial immobilization in nonhost resistance of soybean was found for the *E. carotovora* subsp. *atroseptica*–soybean interaction. Most bacterial cells were surrounded by highly electron-dense material by 48 h after inoculation. The reaction of soybean tissue to heterologous *C. flaccumfaciens* pv. *flaccumfaciens* appeared mixed with bacterial cells occasionally surrounded by fibrillar electron-dense material (possible bacterial extracellular polysaccharide) or more highly electron-dense nonfibrillar material. It did not appear that the immobilization of *C. flaccumfaciens* pv. *flaccumfaciens* by electron-dense material was by itself responsible for restriction of bacterial growth, since most bacterial cells seen were free in the intercellular spaces. Most cells of *B. cereus* located under the electron microscope were covered by highly electron-dense nonfibrillar material, but it is difficult to speculate on the role of bacterial immobilization in the complete inhibition of growth and actual decline of *B. cereus* populations *in vivo* since few bacterial cells were observed.

Although it is possible that a single, as yet undetermined, resistance mechanism is responsible for both host and nonhost resistance to bacteria, the findings reported here and in earlier findings on xanthomonad–soybean interactions reported elsewhere [9,

13] indicate that several mechanisms of resistance may be functioning in soybean. The *C. flaccumfaciens* pv. *flaccumfaciens*-soybean cv. Harosoy interaction studied by the present authors, and the *P. syringae* pv. *syringae*-soybean cv. Merit interaction studied by Érsek & Hevesi [5] should be good candidates for further study. Growth kinetics of these bacteria were similar to those seen for incompatible HR-inducing races of *P. syringae* pv. *glycinea* in soybean leaves, but there was no accompanying stress metabolite accumulation or hypersensitive response. With these two host responses absent, the primary operative resistance mechanism(s) may be more easily identified.

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