

Bacterial growth and phytoalexin elicitation in soybean cell suspension cultures inoculated with *Pseudomonas syringae* pathovars

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Two commonly used media for the culturing of numerous plant tissues, 1-B5 and 1-B5C, were tested for their ability to support bacterial growth. No significant growth of strains representing four of the six major genera of phytopathogenic bacteria occurred in 1-B5 liquid medium, in contrast to 1-B5C, making 1-B5 highly suitable for studies of most phytopathogenic bacteria-plant tissue culture interactions.

Both compatible and incompatible strains of the soybean bacterial leaf pathogen *Pseudomonas syringae* pv. *glycinea* plus strains of the bean pathogens *P. syringae* pv. *phaseolicola* and pv. *syringae*, induced production of the phytoalexin glyceollin in soybean cv. Mandarin cell suspension cultures. Glyceollin production was elicited without a concomitant hypersensitive response (HR). Induced glyceollin production by cell suspension cultures declined as the number of culture transfers increased. There was no expression of resistance to incompatible strains of pv. *glycinea*, pv. *phaseolicola* or pv. *syringae* by the suspension cultured soybean cells. This was indicated by: (1) highest glyceollin production by soybean cell line Sb-2 after inoculation with a compatible pv. *glycinea* strain, (2) greater growth of pv. *syringae* strains than pv. *glycinea* and pv. *phaseolicola* and (3) an incompatible pv. *glycinea* strain grew as well as compatible pv. *glycinea* strains. Of several bacterial strains which elicited an HR on soybean leaves *in vivo*, only pv. *syringae* strain Y30 caused a rapid decline in viability of suspension cultured soybean cells. Various bioassays were used in an attempt to determine if production of the pv. *syringae* phytotoxin syringomycin was responsible.

INTRODUCTION

Pseudomonas syringae pv. *glycinea* (Coerper) Young, Dye & Wilkie, causal agent of bacterial blight of soybean [*Glycine max* (L.) Merr.], was estimated to have caused the largest dollar loss of any procaryotic plant pathogen in the U.S.A. from 1975 through 1977 [38]. The need for study of this pathogen-host interaction is not only indicated by its economic impact, but also because it can serve as a model system for the biochemical basis of race-specific resistance mechanisms [5, 9]. A good correlation between phytoalexin elicitation and soybean-pv. *glycinea* specificity has been observed *in vivo* [33, 35]. At least nine physiologic races of pv. *glycinea* are known which can be distinguished on seven differential soybean cultivars [9, 20, 62]. Resistant reactions are characterized by a hypersensitive response (HR) and induced production of the soybean isoflavonoids glyceollin, coumestrol and daidzein [35] plus additional isoflavonoids [30]. Four isomers of glyceollin have been reported [6, 45, 46].

We chose to use soybean cell suspension cultures as an experimental tool in the

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study of the pv. *glycinea*-soybean interaction. The use of cultured-plant cells for the study of host-parasite interactions has several potential advantages over the use of intact plants [31]. Ease and uniformity of exposure of cultured plant cells to pathogens or pathogen components without wounding of host tissue and ease of separation of biochemical products of the interaction are a few of these advantages.

In vitro cultured plant cells have been successfully used to study various bacterium-plant interactions such as *Agrobacterium*-tobacco [47, 59], *Azospirillum*-sugar cane [4] and *Rhizobium*-soybean [7, 55]. Preliminary to the use of cell suspension cultures as an experimental tool to study the pv. *glycinea*-soybean interaction we needed to determine the response of soybean suspension cells to bacterial challenge.

We have previously reported successful bacterial elicitation of the production of the phytoalexin glyceollin by soybean cv. Mandarin cell suspension cultures [17, 18]. We believe these to have been the first reports of phytoalexin elicitation in cultured plant cells by bacteria.

The studies reported here expand the earlier work [18] on the interaction of pv. *glycinea*, pv. *phaseolicola* (Burk.) Young, Dye & Wilkie and pv. *syringae* van Hall (the latter two being pathogens of *Phaseolus vulgaris* L.) with soybean cv. Mandarin cell suspension cultures. Specifically we examined: (1) bacterial growth in liquid media used routinely as growth media for suspension cultured soybean cells, (2) growth of pv. *glycinea*, pv. *phaseolicola*, and pv. *syringae* in soybean cell suspension cultures and (3) the effect of continued culturing of soybean cell suspensions on phytoalexin production.

Results confirmed our earlier reports of bacterially-induced glyceollin production by soybean cell suspensions without a concomitant HR [17, 18]. In addition, the liquid growth medium 1-B5 [19] (used routinely for culturing soybean cells in suspension) neither supported growth of nor maintained viability of representatives of four genera of phytopathogenic bacteria making it ideally suited for studies of bacteria-soybean cell suspension culture interactions. It was also shown that continued culture of soybean cell suspensions led to decreased phytoalexin production. However, expression of resistance to incompatible pv. *glycinea* or resistance to pv. *phaseolicola* and pv. *syringae* was not obtained.

MATERIALS AND METHODS

Cell suspension cultures

Four separate lines of soybean cv. Mandarin cell suspension cultures (designated Sb-1, Sb-2, Sb-2a and Sb-3) were used. The cell suspension culture Sb-1 was generated from callus (previously designated Sb-1) which had been started from root tissue of cv. Mandarin in 1964 by Dr O. L. Gamborg [19] and had been subcultured ever since. Additional calluses were initiated from epicotyl tissue of cv. Mandarin seedlings in our laboratory as previously described [18]. The suspension cell lines which we designated Sb-2 and Sb-2a were initiated from the same stock callus, but Sb-2a was generated in liquid medium from callus at a later date. Suspension cell line Sb-3 was generated from epicotyl-derived callus from a cv. Mandarin seedling grown at a later time. Cell suspension cultures were generated and maintained in liquid 1-B5 medium [19] as previously described [18] unless otherwise noted.

Bacterial and fungal cultures

Corynebacterium flaccumfaciens pv. *flaccumfaciens* (Hedges) Dowson was obtained from Dr M. Schuster, Department of Horticulture, University of Nebraska, Lincoln, Nebraska, U.S.A., and *Erwinia carotovora* ssp. *atroseptica* strain SR8 (Van Hall) Dye from Dr A. Kelman, Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin, U.S.A.

Pseudomonas syringae pv. *glycinea* (Psg) strains A-29-2 and J3-20-4A were isolated from infected soybean leaves [16]. Psg strain 2159 and *P. syringae* pv. *phaseolicola* (Psp) strain 1134 (originally incorrectly identified as pv. *glycinea* [50]) were obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England. *Xanthomonas campestris* pv. *glycines* (Nakano) Dye strain XP 175 was obtained from Dr M. P. Starr, Department of Bacteriology, University of California, Davis, California, U.S.A. Psg strains A-29-2 and J3-20-4A (both race 4) are compatible with cv. Mandarin, while Psg strain 2159 (race 1) is incompatible with this cultivar [16]. *Pseudomonas syringae* pv. *syringae* (Pss) strains Y30 and HVW3 were obtained from Dr D. J. Hagedorn (Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin, U.S.A.). Pss strain Y30 is compatible with bean cv. Tenderwhite, while Psg strain HVW3 is incompatible [12]. Both Pss strains are incompatible with cv. Mandarin. All pseudomonad strains were maintained on King's Medium B agar (KB) [40] and the others on nutrient dextrose agar (NDA) (Difco nutrient agar supplemented with 10 g l⁻¹ of Difco dextrose and 5 g l⁻¹ of Difco yeast extract, Difco Laboratories, Detroit, Michigan, U.S.A.).

The reaction of intact plants of soybean cv. Mandarin to Psg, Psp and Pss strains, of red kidney bean (W. Atlee Burpee Co., Warminster, Pennsylvania, U.S.A.) pods to Psp strains, and of bean cv. Tenderwhite leaves to Pss strains was tested periodically. For tests on soybean, bacterial inoculum was forcibly sprayed onto abaxial leaf surfaces until water-soaking appeared [16]. For tests on bean pods, bacterial inoculum was injected at three equidistant points along each pod and inoculated pods were incubated in a moist chamber. For tests on bean leaves, bacterial inoculum was gently sprayed onto abaxial and adaxial leaf surfaces and inoculated plants kept under high humidity for 24 h immediately after inoculation. Bacterial inoculum contained approximately 10⁷ colony forming units (c.f.u.) ml⁻¹.

A culture of a *Cladosporium* sp. and of *Geotrichum candidum* Lk. ex Pers. strain F260 were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan, U.S.A.).

Partially purified cell wall glucan elicitor from *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin (Pmg) was provided by Drs P. Albersheim and B. Valent (University of Colorado, Boulder, Colorado, U.S.A.).

Bacterial growth in liquid plant culture media

Two commonly used media for the culturing of soybean and numerous other plant tissues are 1-B5 and 1-B5 amended with casein hydrolysate (1-B5C) [19]. To determine if various bacterial strains could grow in these media, bacterial suspensions were placed into 50 ml of 1-B5 or 1-B5C contained in 250 ml DeLong flasks to give approximate bacterial starting concentrations of 1 to 3 × 10⁷ c.f.u. ml⁻¹. Flasks were then shaken on a rotary shaker at 150 oscillations min⁻¹ maintained at 26 to 27 °C

under constant illumination. Bacterial growth was followed either by standard serial dilution and surface plating techniques or by removing aliquots at intervals and determining optical density at 600 nm.

Bacterial growth in soybean cell suspension cultures

Bacterial inoculum was prepared as described above. Soybean cell cultures from several DeLong flasks were pooled, then redistributed to 250 ml DeLong flasks at 50 ml per flask. In some instances, before redistribution, the pooled soybean cells were removed aseptically from suspension by centrifugation, washed with fresh 1-B5 liquid medium, recentrifuged and then placed into fresh 1-B5 medium. Bacterial inoculum was added to give approximate starting concentrations of 10^8 , 10^5 , or 10^7 c.f.u. ml⁻¹. Bacterial growth was followed by standard serial dilution and surface plating techniques. Culture pH and soybean cell viability (determined by a dye exclusion test with 0.4% trypan blue [54]) were also followed.

Bacterial growth was also followed in Sb-3 cell suspension cultures in 1-B5 liquid media with maltose (20 g l⁻¹) (hereafter designated as 1-B5M) (maltose hydrate, grade 1, Sigma Chemical Company, St. Louis, Missouri, U.S.A.) substituted for sucrose. Soybean cells from 3-day-old cultures (grown in 1-B5 liquid media with sucrose) were washed twice in 1-B5M liquid media by sequentially spooning soybean cells from the parent flask to two flasks containing 50 ml of 1-B5M. The washed Sb-3 cells were then transferred by spooning to 250-ml DeLong flasks containing 30 ml fresh 1-B5M. Bacterial inocula were prepared as described above and bacteria were added to give starting bacterial concentrations of approximately 1×10^7 c.f.u. ml⁻¹. Bacterial growth, soybean cell viability and culture pH were followed as stated above.

In some experiments, glyceollin contents of soybean cells and culture fluid were determined separately along with bacterial growth. Sb-2a cell suspension culture was added to 125-ml DeLong flasks at 25 ml per flask. Bacterial inoculum was added to give approximate starting concentrations of 10^5 or 10^7 c.f.u. ml⁻¹. After 24, 48, 72 and 96 h incubation, soybean cells were removed from suspension by centrifugation and the supernatant fluid collected. Cells were washed once with distilled water recentrifuged and the supernatant fluid collected. The soybean cells were lyophilized. The combined supernatant fluids were extracted three times each with five volumes of chloroform and the combined chloroform layers were evaporated to dryness under N₂. Lyophilized cells were extracted and glyceollin content of cell samples and supernatant fluids were determined as described below.

Phytoalexin induction, isolation, and quantification

Phytoalexin induction by Psg strains A-29-2 and 2159, Psp strain 1134, and Pss strain Y30 *in vivo* was determined by spray-inoculating first trifoliolate leaves of cv. Mandarin with sterile water suspensions of bacteria containing approximately 1×10^8 c.f.u. ml⁻¹ until water-soaking appeared [16]. Approximately 70% of the abaxial leaf tissue had a water-soaked appearance. After 2 to 3 days leaves were extracted for phytoalexins by the facilitated diffusion technique [33]. The dried ethyl acetate extracts were taken up in 1 ml methanol g⁻¹ fresh weight. Twenty-five microlitres of each sample was used to bioassay for activity against *Cladosporium* sp. [37]. Four hundred

microlitres of each sample was then subjected to thin-layer chromatography (t.l.c.) on silica gel plates (250 μm) with fluorescent indicator (Analtech, Newark, Delaware, U.S.A.) and plates were developed in hexane : ethyl acetate : methanol (60 : 40 : 1, v/v). Further procedures were as described below.

Suspension cell cultures of Sb-1, Sb-2, Sb-2a and Sb-3 were used 2 to 4 days (4 to 8 mg dry weight cells ml^{-1} of culture) after transferring cultures to fresh 1-B5 liquid medium. Two to 4-day-old Sb-1 and Sb-3 cell suspension culture were in log phase as determined by dry weight measurements. Growth characteristics of the Sb-2 and Sb-2a cell suspension cultures were not measured. Cell suspensions of each line were pooled and then redistributed to 125-ml DeLong flasks at 25 ml per flask. Bacterial inoculum was prepared by suspending cells of 24 to 48 h cultures grown on KB agar at room temperature in sterile water, washing the cells three times in sterile water, and adjusting the bacterial concentration turbidometrically. Bacterial inoculum (0.25 ml) was added to flasks to give an approximate concentration of 1×10^7 c.f.u. ml^{-1} of culture. Using Psg strain 2159, preliminary experiments showed no decline in viability of bacterial cells kept in sterile water for up to 3 h. The process of washing the bacterial cells through the inoculation of the cell suspension cultures was completed within 1 h. Sterilized preparations of Pmg cell wall glucan in water were added to the soybean cultures at 10 μg dry weight ml^{-1} of culture. Ebel *et al.* [11] reported maximum stimulation of phenylalanine ammonia-lyase activity in soybean suspension cell cultures by Pmg cell wall glucan after addition of 1 to 8 μg ml^{-1} .

After 24 or 48 h incubation (48 h being the approximate time of maximum glyceollin accumulation *in vivo* as reported by Keen & Kennedy [35]), the entire flask contents were lyophilized, ground to a fine powder, twice extracted by vigorous stirring for 5 min with 70 ml ethyl acetate : methanol (9 : 1, v/v) g^{-1} dry weight [37], and filtered. The combined extracts were dried under a stream of N_2 and taken up in 1 ml 95% ethanol g^{-1} dry weight. Fifty microlitres of each sample was used in a t.l.c. bioassay designed to detect activity against *Cladosporium* sp. [37]. The remainder of the sample was subjected to t.l.c. on silica gel plates (250 μm) with fluorescent indicator and developed in hexane : ethyl acetate : methanol (60 : 40 : 1, v/v).

Developed t.l.c. plates were examined under 254 nm and 350 nm light. Fluorescence-quenched, yellow fluorescent, or blue fluorescent areas corresponding to standards of glyceollin, daidzein and coumestrol (Eastman Kodak, Rochester, New York, U.S.A.), respectively, were scraped from the plates, packed into small glass columns, and eluted with 9 ml of 95% ethanol. Eluates were dried under a stream of N_2 , the residue taken up in 2 ml 95% ethanol, and the u.v. absorbance spectrum of each sample determined.

Glyceollin concentration was calculated from absorption at 285 nm and the molar extinction coefficient of 10 300 [2] or by high-performance liquid chromatography (h.p.l.c.). A Waters Associates (Milford, Massachusetts, U.S.A.) h.p.l.c. system with a Model U6K injector attached to a Model M-6000A chromatography pump and a micro-Porasil column with a 10 μm average particle diameter was used. After determining u.v. absorptions, samples were dried under a stream of N_2 and the residue taken up in 100 μl 95% ethanol. Twenty-five microlitre aliquots of each sample were used for injection. Glyceollin was eluted with isopropanol : hexane (5 : 95, v/v) at a

flow rate of 2 ml min⁻¹. Absorbance at 285 nm was followed with a Model 450 variable wavelength detector.

Daidzein and coumestrol concentrations were determined by h.p.l.c. as described above with minor adjustments as noted below. Dried culture extracts were taken up in 50 µl 95% ethanol and 20 µl aliquots were used for injection. Daidzein was eluted with isopropanol : hexane (10 : 90, v/v) and absorbance was monitored at 260 nm. Coumestrol was eluted with isopropanol : hexane (3 : 97, v/v) and absorbance was monitored at 343 nm. The identification of glyceollin and daidzein in selected samples was further confirmed by mass spectrometry using a Hewlett-Packard 5992B GC/MS and a Varian MAT 311A at 70 Ev with solid probe sample introduction, respectively.

The ability of heat-killed Psg strain 2159 to elicit glyceollin production in Sb-3 cell suspension culture was tested by following the above procedure except the bacterial suspensions were heated for 10 min at 100 °C before addition to soybean cell suspension cultures. Samples from the heated bacterial suspensions were surface-plated on NDA as a viability check. No bacterial growth occurred. Soybean cell suspension cultures were extracted after 48 h and glyceollin content determined as described above.

Phytotoxin bioassays

The production of phytotoxin by Psg, Psp and Pss strains was sought on agar medium and in soybean cell suspension cultures. All the bacterial strains were spot inoculated onto PDA plus 0.4% Difco casamino acids, an optimal agar medium for testing Pss strains for fungal inhibitory activity due to the presence of phytotoxins [58]. Inoculated plates were incubated for 5 days at room temperature and then sprayed with a sterile water suspension of *G. candidum* strain F260 arthrospores (approximately 10⁷ spores ml⁻¹). *Geotrichum candidum* strain F260 is very sensitive to the toxic effects of the Pss toxin syringomycin [58]. Plates were observed up to 8 days of additional incubation.

Sterile culture filtrates were obtained from Sb-1 cell suspension cultures, which had been inoculated with Pss strain Y30 or with the Psg and Psp strains and incubated for 72 h, by filtering cultures through 0.45 µm Nalgene filter units (Nalge Company, Rochester, New York, U.S.A.). The sterile filtrates were tested for chlorosis-inducing activity on leaves of soybean cv. Harosoy and/or bean cv. Tenderwhite by the droplet-prick method [49] and for inhibition of *G. candidum* strain F260 by spotting 5 µl samples on PDA previously sprayed with an arthrospore suspension [58].

RESULTS

Pathogenicity tests

Psg strains A-29-2 and J3-20-4A were pathogenic towards soybean cv. Mandarin producing water-soaked leaf lesions within 4 days. Psg strain J3-20-4A, but not strain A-29-2, also induced systemic toxemia, evident by 7 days after inoculation presumably due to production of the bacterial toxin coronatine [51]. The compatible Psg strains did not change in virulence toward leaves of cv. Mandarin over the experimental period. Psg strain 2159 and Psp strain 1134 elicited an HR characterized by browning of the leaf laminae visible in 24 to 48 h. Strain 2159 was pathogenic towards soybean cv. Flambeau, typical of race 1 strains [9] and Psp strain 1134 gave water-soaked lesions on bean pods within 5 days after incubation.

Pss strain Y30 was highly virulent on leaves of bean cv. Tenderwhite giving water-soaked lesions within 4 days after inoculation. Within 1 week systemic toxemia, presumably due to the bacterial toxin syringomycin [21] was evident. Pss strain HVW3 was not pathogenic towards cv. Tenderwhite in agreement with the report by Ercolani *et al.* [12]. Pss strains Y30 and HVW3 both elicited an HR characterized by browning in 24 to 48 h on cv. Mandarin.

The Pmg cell wall glucan was tested periodically for activity on soybean cv. Harosoy cotyledons using the procedure of Ayers *et al.* [2]. Its glyceollin inducing ability did not decline during the experimental period.

Bacterial growth in liquid plant culture media

Representatives of four of the six major genera of phytopathogenic bacteria were tested for their growth in 1-B5 or 1-B5C liquid media. The liquid medium 1-B5 supported little growth and did not maintain viability of Psg strains A-29-2, 2159 and J3-20-4A and Psp strain 1134 (Fig. 1). Growth of Pss strain Y30, *E. carotovora* ssp. *atroseptica* strain SR8, *C. flaccumfaciens* pv. *flaccumfaciens* and *X. campestris* pv. *glycines* strain 175 also was very low in 1-B5 liquid media (Table 1). However, in the presence of casein hydrolysate (1-B5C), all bacterial strains exhibited strong growth (Table 1). Thus, 1-B5 but not 1-B5C is an excellent medium to study the interaction of various phytopathogenic bacteria with plant cell suspension cultures.

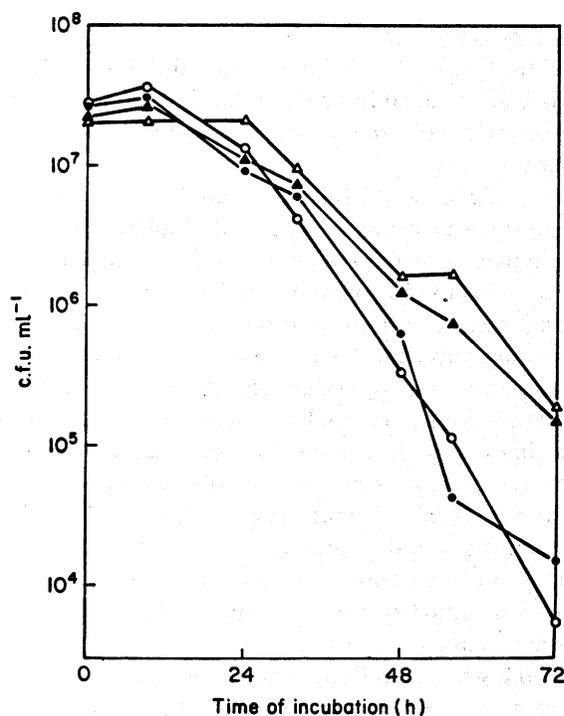


FIG. 1. Growth of *P. syringae* pv. *glycinea* (Psg) and pv. *phaseolicola* (Psp) in 1-B5 liquid media. ●, Psg strain A-29-2; ○, Psg strain 2159; ▲, Psg strain J3-20-4A; Δ, Psp strain 1134.

TABLE 1
Bacterial growth in 1-B5 and 1-B5C liquid media

Bacterium ^a	Strain	Medium	O.D. 660 nm				
			0 h	6 h	24 h	48 h	72 h
<i>E. carotovora</i>	SR8	1-B5	0.010 ^b	— ^c	0.130	0.026	0.060
		1-B5C	0.022	0.330	>2.0	>2.0	—
<i>C. flaccumfaciens</i>	—	1-B5	0.005	—	0.021	0.032	0.060
		1-B5C	0.010	0.039	1.015	1.390	—
<i>P. syringae</i>	2159	1-B5	—	—	—	—	—
		1-B5C	0.007	0.047	1.762	1.945	—
<i>pv. glycinea</i>	A-29-2	1-B5	—	—	—	—	—
		1-B5C	0.018	0.100	1.715	1.704	—
	J3-20-4A	1-B5	—	—	—	—	—
		1-B5C	0.007	0.052	1.737	1.700	—
<i>pv. phaseolicola</i>	1134	1-B5	—	—	—	—	—
		1-B5C	0.015	0.093	1.730	1.861	—
<i>pv. syringae</i>	Y30	1-B5	0.010	—	0.019	0.017	0.045
		1-B5C	0.015	0.135	1.803	1.695	—
<i>X. campestris</i>	175	1-B5	0.012	—	0.022	0.054	0.058
		1-B5C	0.013	0.065	>2.0	>2.0	—

^a Starting bacterial concentrations were 1 to 3×10^7 c.f.u. ml⁻¹. Culture flasks were shaken at 26 °C.

^b Values represent averages of data from two separate experiments.

^c (—) denotes not determined.

Bacterial growth in cell suspension cultures

Growth of Psg strains A-29-2, J3-20-4A (both compatible) and 2159 (incompatible) plus Psp strain 1134 was determined in Sb-1, Sb-2a and Sb-3 cell suspension cultures with or without transferring cultured soybean cells to fresh 1-B5 liquid medium immediately before inoculation.

Psg strains A-29-2, J3-20-4A and 2159 grew equally well over a 96-h incubation period in cultures of all three soybean cell lines with doubling times ranging between 3 to 6 h. Data from a typical growth experiment for Psg strains A-29-2 and 2159 using starting bacterial concentrations of approximately 10^7 c.f.u. ml⁻¹ is shown in Fig. 2(a). When starting bacterial concentrations of approximately 10^7 c.f.u. ml⁻¹ were used maximum bacterial populations (2 to 4×10^9 c.f.u. ml⁻¹) were reached in 60 to 70 h. When starting concentrations of approximately 10^5 or 10^3 c.f.u. ml⁻¹ were used maximum bacterial populations were reached after 70 h [Fig. 3(a)]. Psp strain 1134 also showed doubling times between 3 to 6 h. but often bacterial populations reached stationary phase more rapidly and maximum populations of only 5 to 10×10^8 c.f.u. ml⁻¹ were reached (not shown). Transferring soybean cells to fresh 1-B5 liquid media before inoculation did not alter growth of Psg or Psp strains.

When the 1-B5 liquid medium containing sucrose is used in the study of bacterial growth in soybean cell suspension cultures, the sucrose is available to the bacteria as a carbon source. Under such conditions, the bacteria may not need to obtain their source of carbon from the soybean cells as required *in vivo*. Therefore, we wanted to identify a carbon source to replace sucrose in the 1-B5 medium that would sustain viability of the cultured soybean cells and yet not be utilizable by the bacteria. Maltose met these criteria as noted below.

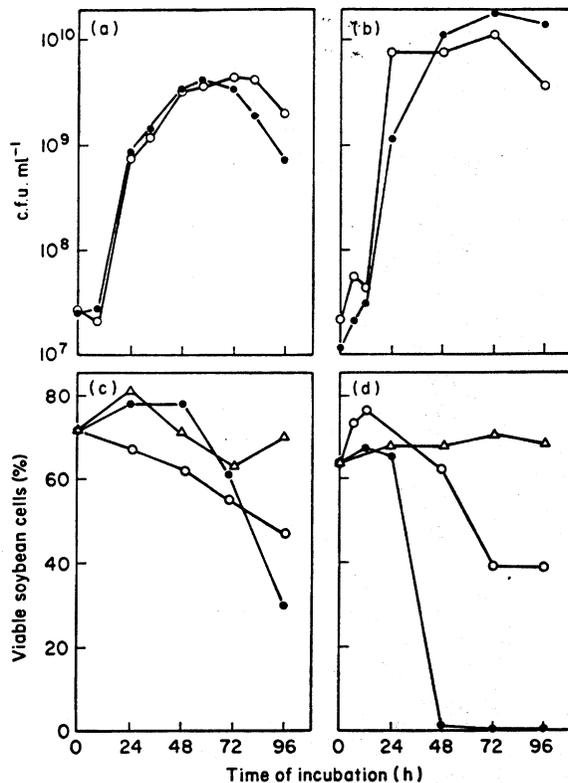


FIG. 2. Growth of *P. syringae* pv. *glycinea* (Psg) and pv. *syringae* (Pss) in soybean cell suspension cultures using starting bacterial concentrations of approximately 10^7 c.f.u. ml⁻¹ and the effect on soybean cell viability. Growth curves for (a) Psg and (b) Pss strains using soybean cell lines Sb-1 (after 50 culture transfers in our laboratory) and Sb-3 (after eight culture transfers), respectively. (a) ●, A-29-2; ○, 2159. (b) ●, Y30; ○, HVW3. (c) and (d) Effect of bacterial growth on soybean cell viability. (c) ●, A-29-2; ○, 2159; Δ, control. (d) ●, Y30; ○, HVW3; Δ, control.

When maltose (20 g l^{-1}) was substituted for sucrose in the 1-B5 medium (designated 1-B5M) viability of Sb-3 suspension cells growing alone did not decrease over a 5-day period. In addition, Psg strains A-29-2 and 2159 and Psp strain 1134 were unable to utilize maltose as their sole source of carbon. This was shown by using the mineral salts medium of Stanier [61] as altered by Misaghi & Grogan [48]. Filter sterilized maltose or sucrose (20 g l^{-1}) were added to the cooled medium containing 15 g l^{-1} of agar. Bacterial strains were each spotted onto at least three separate plates of solidified media and plates were then incubated at approximately 24°C for 21 days. After 21 days no growth of the three bacterial strains on the maltose containing medium was evident. All three bacterial strains grew (colony diameter of 0.5 cm) on the sucrose containing medium. No bacterial growth occurred in 1-B5M liquid medium alone.

Psg strains A-29-2 and 2159 and Psp strain 1134 were tested for growth in Sb-3 cell suspension cultures in 1-B5M liquid media. Bacterial growth and the effect on

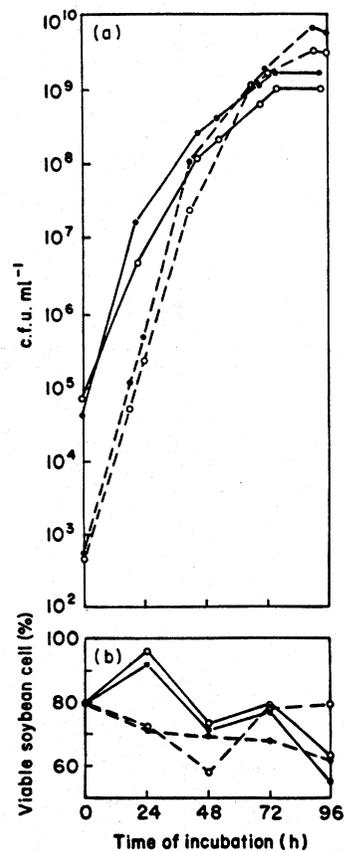


FIG. 3. Growth of *P. syringae* pv. *glycinea* (Psg) in Sb-3 soybean cell suspension cultures using starting bacterial concentrations of approximately 10^3 (broken lines) (Sb-3 used after 23 culture transfers) or 10^5 (solid lines) (Sb-3 used after 28 culture transfers) c.f.u. ml⁻¹ and the effect on soybean cell viability. (a) Growth curves for Psg strains A-29-2 (●) and 2159 (○). (b) Effect of bacterial growth on soybean cell viability for Psg strains A-29-2 (●) and 2159 (○).

soybean cell viability did not differ significantly from results using 1-B5 media (not shown).

Growth of Pss strains Y30 and HVW3 was also determined in Sb-3 cell cultures where soybean cells were transferred to fresh 1-B5 liquid medium immediately before use. Doubling time for both Pss strains was approximately 2 h and maximum bacterial concentrations of approximately 1×10^{10} c.f.u. ml⁻¹ were reached in 72 h [Fig. 2(b)].

Along with growth of the Psg, Psp and Pss strains, pH of the culture was followed. The pH of uninoculated control cultures usually stayed within 0.5 pH unit of the starting pH (approximately 6.0) over the 96 h incubation period. The effects of bacterial inoculation on culture pH were variable, but culture pH usually stayed

within one pH unit of control values during the 96 h incubation period. Occasionally, up to 2 pH units difference was noted with pH extremes of 4.2 and 7.7. No relationship between pH changes with the amount of glyceollin produced or with changes in soybean cell viabilities was found.

Effect of glucan and bacteria on soybean cell viability

Treatment of Sb-2, Sb-2a and Sb-3 cell suspension cultures with Pmg cell wall glucan caused darkening of the soybean cells within 24 h. No such darkening effect from the glucan occurred up to 48 h (the longest incubation period tested after addition of the glucan) with Sb-1 cell suspension cultures. Staining with trypan blue showed no evidence of an adverse effect on soybean cell viability of any of the four lines by the Pmg cell wall glucan after 24 or 48 h incubation.

Usually, Psg strains A-29-2, 2159 and J3-20-4A and Psp strain 1134 reduced viability of the Sb-1, Sb-2a and Sb-3 cultured soybean cells over a 96 h incubation period [Figs 2(c), 3(b)]. The most consistent adverse effect by a Psg or Psp strain occurred when Psg strain A-29-2 was inoculated into Sb-1 cell suspension culture. From initial soybean cell viabilities of approximately 70%, viability was reduced to 8 (not shown) to 30% [Fig. 2(c)].

Pss strains HVW3 and Y30 also reduced soybean cell viability over a 96 h incubation period [Fig. 2(d)], but the effect of strain Y30 was much more pronounced. Pss strain Y30 caused a >95% reduction of soybean cell viability within 24 to 48 h after inoculation into cultures of all four soybean cell lines as reported earlier for lines Sb-1 and Sb-2 [18]. In recent experiments Pss strain Y30 caused a >95% reduction in soybean cell viability within 15 h after inoculation.

Heat-killed (100 °C, 10 min) Pss strain Y30 did not affect viability of soybean suspension cells.

TABLE 2

Phytoalexin production in soybean cv. Mandarin leaves 2 to 3 days after bacterial inoculation

Eliciting agent ^a	Interaction ^b	$\mu\text{g g}^{-1}$ Fresh weight		
		Glyceollin	Daidzein	Coumestrol
H ₂ O	—	0 ^c	0	0
<i>P. syringae</i>				
<i>pv. glycinea</i>				
A-29-2	C	2	0	0
2159	I	105	10	32
<i>pv. phaseolicola</i>				
1134	I	32	4	10
<i>pv. syringae</i>				
Y30	I	63	19	12

^a First trifoliolate leaves of cv. Mandarin were spray-inoculated with bacterial suspensions containing approximately 1×10^8 c.f.u. ml⁻¹ until water-soaking was evident.

^b C = compatible interaction; I = incompatible interaction characterized by a hypersensitive response.

^c Values represent averages of data from two experiments and are uncorrected for extraction efficiency.

Phytoalexin production

The presence of glyceollin in the leaf and cell suspension culture extracts was confirmed by toxicity towards *Cladosporium* sp., behaviour in t.l.c., u.v. spectra, h.p.l.c. and GC-mass spectrometry [36, 37].

In vivo the three incompatible bacterial strains tested (Psg strain 2159, Psp strain 1134, and Pss strain Y30) all elicited higher production of the soybean isoflavonoids glyceollin, coumestrol and daidzein than did the compatible Psg strain A-29-2 (Table 2). However, the levels of glyceollin, coumestrol and daidzein were much lower (approximately 10 to 40-fold for glyceollin and coumestrol and 100 to 500-fold for daidzein) than those reported by Keen & Kennedy [35] for the interaction of soybean leaves with an incompatible Psg strain. The isomeric composition of the leaf glyceollin as determined by h.p.l.c. was 60%-III, 31%-II and 9%-I. Recoveries of glyceollin standards added to uninoculated leaves sitting in 40% ethanol just before vacuum infiltration were approximately 60%.

All four bacterial strains tested elicited glyceollin production by the Sb-2 cell suspension culture (Table 3). Compatible Psg strain A-29-2 elicited more glyceollin

TABLE 3
Elicitation of glyceollin in soybean cv. Mandarin (line Sb-2) cell suspension cultures

Eliciting agent	Hours of incubation	Glyceollin production v. number of culture transfers					
		5 ^a	8	14	17	32	44
Pmg glucan ^b	24	103 ^c	13	0	16	0	0
<i>P. syringae</i>							
<i>pv. glycinea</i> ^d							
A-29-2	24	27	37	0	0	8	36
	48	333	140	150	35	127	21
2159	24	— ^e	12	0	0	—	—
	48	—	15	0	0	—	—
<i>pv. phaseolicola</i>							
1134	24	45	18	0	0	—	—
	48	144	34	27	0	—	—
<i>pv. syringae</i>							
Y30	24	69	10	0	0	—	—
	48	21	11	0	0	—	—

^a Suspension cultures were transferred every 5 to 7 days.

^b Cell wall glucan from *P. megasperma* f. sp. *glycinea* added at 10 µg dry weight ml⁻¹ of culture.

^c Values (µg glyceollin g⁻¹ dry weight) represent data from single flasks. Glyceollin values were determined by A₂₈₅ and ε = 10 300. Control A₂₈₅ values were subtracted from treatment values. Values are uncorrected for extraction efficiency.

^d Bacterial inoculum was added at approximately 1 × 10⁷ c.f.u. ml⁻¹.

^e (—) = not determined.

than the incompatible Psg strain 2159 and Psp strain 1134 after a 48 h incubation period. Strain A-29-2 also elicited more glyceollin production than the Pmg cell wall glucan at the levels of bacteria and glucan tested. The isomeric composition of the glyceollin produced by Sb-2 cell suspension cultures varied with isomers I and II being predominant (accounting for approximately 80 to 90%) and isomer III present in lesser amounts (10 to 20%).

A distinct trend towards decreased glyceollin induction with the number of Sb-2 cell suspension culture transfers was noted for all treatments (Table 3). After the seventeenth culture transfer only Pmg cell wall glucan and Psg strain A-29-2 elicited detectable amounts of glyceollin. Extracts from untreated Sb-2 cell cultures after equivalent numbers of culture transfers showed a low level of absorbance at 285 nm but no peak at this wavelength. The absence of glyceollin in the control flasks was further confirmed by additional tests as stated above.

Glyceollin content in cultured soybean cells and in the culture fluid was determined separately over 96 h after inoculation of line Sb-2a and Sb-3 cell suspension cultures with either approximately 10^7 (Fig. 4) or 10^5 c.f.u. ml^{-1} of Psg strain 2159.

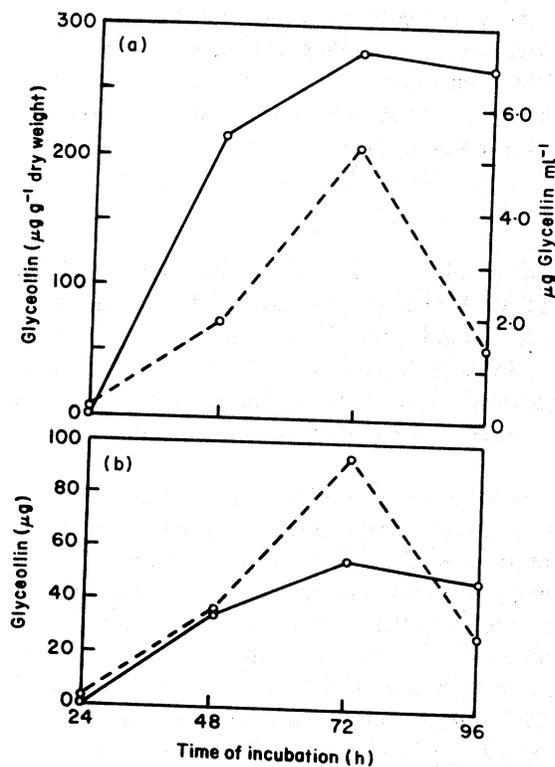


FIG. 4. Glyceollin content in soybean cell suspension cultures (line Sb-2a after ten culture transfers) inoculated with approximately 1×10^7 c.f.u. ml^{-1} of *P. syringae* pv. *glycinea* strain 2159. Glyceollin content of soybean cells (—) and culture fluid (---) were determined separately and are reported as $\mu\text{g g}^{-1}$ dry weight soybean cells and $\mu\text{g ml}^{-1}$ culture fluid (a) or as total μg (b). Values are uncorrected for extraction efficiency.

At both starting concentrations, maximum glyceollin content in the soybean cells and in the culture fluid was reached by 72 h after inoculation at the time of maximum or near maximum bacterial populations [Fig. 2(a)]. After 72 h glyceollin levels declined.

After the study was begun on the effect of continued culturing of soybean cell

suspensions on elicited glyceollin production, it was determined that only approximately 25% of glyceollin added to untreated soybean cell suspension cultures was recovered after extraction. Since continuity for this study was required, the extraction method was continued in spite of the low recoveries. The reason(s) for the low recoveries is not known. All isoflavonoid values reported herein were uncorrected for extraction efficiency unless noted otherwise.

Heat-killed Psg strain 2159 in contrast to non-heat-treated bacteria did not elicit glyceollin production in soybean cell suspension cultures.

The presence of daidzein in the cell suspension cultures was confirmed by behaviour in t.l.c., u.v. spectra and mass spectrometry [36]. Variable amounts (0 to 150 $\mu\text{g g}^{-1}$ dry weight) were found in both uninoculated and bacteria-inoculated Sb-2 cell suspension cultures.

Coumestrol (50 $\mu\text{g g}^{-1}$ dry weight culture) was detected in a single cell suspension culture of line Sb-1 24 h after addition of Pmg cell wall glucan. The presence of coumestrol was confirmed by behaviour in t.l.c. and the u.v. spectra [36]. Several other identically treated Sb-1 cell suspension cultures did not contain coumestrol. Sb-2, Sb-2a and Sb-3 cell suspension cultures were not found to produce coumestrol in response to the Pmg cell wall glucan or Psg, Psp and Pss strains.

Phytotoxin bioassays

On PDA plus casamino acids only the two Pss strains showed inhibitory activity against *G. candidum* strain F260. Pss strain Y30 and HVW3 caused zones of complete inhibition of fungal growth around bacterial colonies; 23 mm from the edge of colonies of strain Y30 and 63 mm for strain HVW3. None of the Psg strains or the Psp strain were inhibitory towards *G. candidum* on this medium. This fungus is highly sensitive to the presence of both of the Pss toxins syringomycin [58] and syringotoxin [23].

An additional assay commonly used to detect the presence of phytotoxins is to make a small wound in the leaf tissue underneath a droplet of the test solution [49]. When sterile culture filtrates from the 72 h interaction of the three Psg strains, the Psp strain and Pss strain Y30 with cultures of cell line Sb-1 were tested in this manner on leaves of bean cv. Tenderwhite, only the culture filtrate from the Pss strain Y30 and Sb-1 interaction gave a definite circular chlorotic area around the prick point. The culture filtrate from Psg strain A-29-2 and Sb-1 caused very slight chlorosis. Sterile culture filtrates from the 72 h interaction of Psg strains A-29-2 and Psp strain 1134 with Sb-1 were also tested on leaves of soybean cv. Harosoy with no resultant chlorosis after 5 days incubation. When all of the above sterile culture filtrates were spotted (5 μl) on PDA agar pre-sprayed with an arthrospore suspension of *G. candidum* strain F260, only the culture filtrate from the Pss strain Y30 and Sb-1 interaction showed activity causing a reduced but not completely inhibited zone of fungal growth.

The presence of a phytotoxic factor in culture filtrates obtained after 24 h incubations of Pss strain Y30 in 1-B5 (no bacterial growth) or 1-B5C (strong bacterial growth) (Table 1) liquid media or in Sb-1 cell suspension cultures after 24 or 48 h (complete killing of the Sb-1 cells occurred within 24 h) was tested for by the following procedure. After incubation, cultures were filtered through 0.45- μm filters and viable Sb-1 cells were added to the sterile culture filtrates. A small amount of each

culture filtrate was withheld for testing for phytotoxicity on bean cv. Tenderwhite leaves. None of the sterile culture filtrates proved to be toxic to the freshly added Sb-1 cells up to 72 h of incubation. When tested on bean leaves, only the sterile culture filtrate from the 48 h interaction of Sb-1 and Pss strain Y30 caused a chlorotic zone around the prick points. The sterile culture filtrates obtained from the 24 h interaction of Sb-1 suspension cells and Pss strain Y30 and the culture filtrates from the 24 h incubations of Pss strain Y30 alone in 1-B5 or 1-B5C were all inactive.

DISCUSSION

The commonly used plant tissue culture liquid growth medium 1-B5 should be highly suitable for studies of most phytopathogenic bacteria-plant tissue culture interactions. Representatives of four of the six major genera of phytopathogenic bacteria were tested and found to be unable to grow in liquid 1-B5 media alone. No bacteria representative of the agrobacteria were tested but Matthyse *et al.* [47] recently reported *Agrobacterium tumefaciens* (Smith & Townsend) Conn was unable to grow in the tissue culture growth medium of Murishige and Skoog [52]. Thus, appropriate tissue culture media for use in the study of phytopathogenic bacteria-plant tissue culture interactions are already available.

Maltose should be substituted for sucrose for the study of phytopathogenic bacteria-plant tissue culture interactions where the bacteria cannot utilize maltose as a carbon source (as with the phytopathogenic oxidase-negative, arginine dihydro-lase-negative fluorescent pseudomonads [57]) and the plant tissue cultures can survive with maltose as their primary carbon source. This situation requires the bacteria to obtain additional nutrients from the plant cells, thus more closely mimicking the *in vivo* interaction.

The results of this study confirm our earlier reports [17, 18] of bacterially-induced glyceollin production in suspension cultured soybean cells. Kennedy *et al.* [39] had reported that soybean callus cultures did not respond to inoculation by incompatible Psg strains or by bacteria pathogenic towards other hosts with an HR or production of soybean phytoalexins. The isomeric composition of glyceollin produced by soybean cell suspension cultures (primarily isomers I and II) more closely approximates the isomeric composition of glyceollin produced by soybean seed (primarily isomer I [15]) than that of glyceollin produced by soybean leaf tissue (primarily isomer III) [15, 30]. When the maximum glyceollin values elicited in cv. Mandarin cell suspension cultures and the values elicited *in vivo* are adjusted for extraction efficiencies based on recoveries of added glyceollin standards and the *in vivo* values adjusted for a water content of the leaf tissue of 87% on a fresh weight basis (determined by drying leaves to a constant weight), the levels of glyceollin (expressed as $\mu\text{g g}^{-1}$ dry weight) produced *in vivo* and in cell suspension cultures in response to inoculation with incompatible Psg, Psp and Pss strains were similar (Table 4). Glyceollin elicited by the compatible Psg strain A-29-2 was much greater in cell suspension culture than *in vivo* (Table 4). Elicitation of similar levels of the phytoalexin medicarpin occur after inoculation of jackbean callus or detached jackbean hypocotyls with fungal spores [25] and Ebel *et al.* [11] obtained similar levels of glyceollin in soybean suspension cultures treated with Pmg cell wall glucan as found in Pmg cell wall glucan-treated soybean hypocotyls [2]. However, Keen & Horsch

TABLE 4
Comparison of glyceollin accumulation after bacterial inoculation of soybean cv. Mandarin cell suspension cultures or leaves

Eliciting agent	Inoculated	Interaction ^a	Time after inoculation	Glyceollin ^b ($\mu\text{g g}^{-1}$ dry wt.)
<i>P. syringae</i> pv. <i>glycinea</i> A-29-2	Sb-2 cell suspension (after five transfers)	C	48 h	1332
	Leaves	C	2-3 days	25
2159	Sb-2a cell suspension (after ten transfers)	I	72 h	600
	Leaves	I	2-3 days	1346
pv. <i>phaseolicola</i> 1134	Sb-2 cell suspension (after five transfers)	I	48 h	576
	Leaves	I	2-3 days	408
pv. <i>syringae</i> Y30	Sb-2 cell suspension (after five transfers)	I	24 h	276
	Leaves	I	2-3 days	808

^a C Denotes a compatible interaction; I denotes an incompatible interaction characterized by a hypersensitive response.

^b Glyceollin values were adjusted according to a 25% recovery of glyceollin from cell suspension cultures, a 60% recovery of glyceollin from leaves and a water content of 87% on a fresh weight basis for leaves.

[34] found much lower glyceollin production by calluses than hypocotyls of a resistant soybean cultivar after inoculation with *P. megasperma* f. sp. *glycinea*.

In the present study, no clear induced production of the soybean isoflavonoids coumestrol and daidzein was found in bacterially-inoculated soybean cell suspension cultures in contrast to what occurred *in vivo*.

Even though similar levels of glyceollin were elicited by incompatible Psg and Psp strains in cell suspension cultures and *in vivo*, no HR occurred in cell suspension culture. This may indicate that either glyceollin accumulation *per se* is not responsible for the HR *in vivo* due to the phytotoxic nature of glyceollin [42] or that in liquid media the soybean cells can "escape" the phytotoxic effect of glyceollin through its excretion into the culture media thus greatly diluting its effect. However, usually a slow decline of soybean cell viability in bacterially-inoculated cell suspension cultures occurred. The host cells that did die may have been the ones that produced glyceollin.

The time course studies with Psg strain 2159 and soybean cell line Sb-2a indicated maximum glyceollin levels were reached by 72 h after inoculation. Since time course studies of *in vivo* glyceollin accumulation were not carried out with cv. Mandarin and Psg strain 2159 (a race 1 strain) no direct comparison can be made. However, Keen

& Kennedy [35] reported glyceollin accumulated to maximum levels approximately 50 h after inoculation of soybean cv. Harosoy with an incompatible Psg race 1 strain. A similar lag in glyceollin production appears to occur in soybean cell suspension cultures inoculated with Pmg cell wall glucan (glyceollin accumulation required a minimum of 40 h after treatment) [11] when compared to similarly treated soybean cotyledons and hypocotyls (maximum glyceollin accumulation by 30 h) [2].

Within 24 to 48 h after inoculation of Sb-2, Sb-2a and Sb-3 soybean cell suspension cultures with all Psg and Psp strains, no significant reduction of soybean cell viability occurred even though glyceollin production in culture was detected. *In vivo*, leaves of soybean cv. Mandarin inoculated with incompatible Psg and Psp strains showed an HR within this time period. The Pmg cell wall glucan, though causing darkening of the cultured soybean cells, did not decrease soybean cell viability but did induce glyceollin production. Thus, rapid death of the suspension cultured cells characteristic of the HR *in vivo* was not necessary for glyceollin production. There have been previous reports of production of the pterocarpanoid phytoalexin phaseollin without an HR in bean cell suspension cultures [27] and in live bean endocarp [53]. Pisatin also accumulated in chemically-treated pea endocarp [10] without the HR.

There was no indication that the soybean cells in suspension reacted to the Psg strains in a race-specific manner. In addition to not obtaining an HR in any of the Psg-soybean cell suspension culture interactions, at least comparable glyceollin production occurred in Sb-2 cell cultures after inoculation with the compatible Psg strain as with the incompatible Psg, Psp and Pss strains.

Results of the bacterial growth studies also indicated no resistance expressed towards Psg, Psp and Pss at any of the initial bacterial concentrations studied. In fact, the incompatible Pss strains reached higher populations than the compatible Psg strains. The compatible or incompatible nature of bacterial strains did not appear to affect growth in soybean cell suspension cultures under the conditions employed. The substitution of maltose, which was shown to be non-utilizable by Psg and Psp strains, for sucrose in 1-B5 media did not adversely affect growth of Psg or Psp strains in soybean cell suspension cultures. This finding plus the fact that the bacteria grew very little in 1-B5 liquid medium (with sucrose—a utilizable sugar) alone indicates that, as *in vivo*, the bacteria could obtain sufficient carbon plus additional required nutrients from the soybean cells. Sufficient carbon may have been obtained by: (1) utilization of host cell released carbohydrates or arabinogalactan glycoproteins [43], (2) bacterial enzymatic cleavage of complex carbohydrates at the soybean cell surface, or (3) induced leakage of carbohydrates from the host internal cellular carbohydrate pool by bacterial products affecting membrane permeability. Rapid death of the cultured soybean cells was only in response to inoculation with incompatible Pss strain Y30 and not with incompatible Pss strain HVW3 or the incompatible Psg and Psp strains. The two Pss strains, Psg strain 2159 and Psp strain 1134 induced an HR on soybean cv. Mandarin leaves, however. The maximum level of glyceollin (approximately 5 to 10 $\mu\text{g ml}^{-1}$) found in the culture fluid during bacterial growth in cell suspension culture was lower than that found to affect growth of Psg *in vitro* (12.5 to 400 $\mu\text{g ml}^{-1}$) [14, 15, 35].

There could be several reasons for the lack of a HR in the soybean cell suspension cultures. Plant cells in suspension culture may have inherent difficulty in expressing

a traditional HR to bacterial inoculation. Cook & Stall [8] reported that keeping pepper leaf tissue in a water-soaked condition inhibits the HR to *X. campestris* pv. *vesicatoria* (Doidge) Dye. Also, suspending an HR-inducing strain of pv. *vesicatoria* in water agar and using this suspension to inoculate pepper leaves also inhibited the HR, but not the susceptible response [60]. Such studies suggest that an extended bacterium-host cell contact (but not necessarily active bacterial immobilization at the host cell wall) may be necessary for elicitation of the HR. The usual 2 to 4 h induction period for a bacterially-induced HR [13, 41] may be the time needed for the transfer of HR eliciting factors during close cell to cell contact.

In cell suspension cultures, a prolonged period of close bacterial-host cell contact may not occur where cell to cell contact is not mediated by active binding mechanisms. Fett & Jones [13] found no evidence of active immobilization of Psg in soybean intercellular leaf spaces. *Agrobacterium tumefaciens* (Smith and Town.) Conn, whose specific attachment *in vivo* to host cell walls has been well documented [44], was also reported to attach to tobacco callus [59] resulting in agglutination of suspension cultured plant cells [47]. No clear agglutination was seen in the Psg, Psp or Pss-suspension cultured soybean cell interactions.

Secondly, host cell contact appears to be needed for initiation of an HR after inoculation with a local lesion strain of tobacco mosaic virus [32]. This may also be the case for a bacterially-induced HR. Host cell suspension cultures may lack the necessary degree of cell to cell contact or intercellular communication.

In this and our previous study [18], the Sb-1 cell line, which has been in continuous culture for many years, did not produce glyceollin after addition of biotic or abiotic elicitors. However, recently in our laboratory addition of high levels of the potato stress metabolite katahdinone occasionally induced glyceollin production by Sb-1 [63], but this was not readily reproducible. Thus, it appears that the Sb-1 cell line has not lost the pathway for producing glyceollin but rather the pathway is more difficult to induce. Glyceollin production by cell line Sb-2 decreased with increasing numbers of culture transfers and this may also be due to an increased difficulty in induction rather than a loss of a metabolic pathway. Reduced phytoalexin production by tissue cultured cells with increased time in culture has been reported previously. Bailey [3] found that pea callus cultures showed a marked reduction in ability to produce pisatin after 18 months in culture. Other workers [24] routinely screen stock callus cultures for phytoalexin production and propagate only those calluses still competent in this ability. Instability of phytoalexin production by suspension cultured plant cells might be expected to be greater than for plant calluses because of differences in generation time.

We have not yet been successful in determining the cause of rapid death of soybean suspension cells when exposed to Pss strain Y30. If this was due to an HR, it is difficult to explain why Pss strain HVW3 and the incompatible Psg and Psp strains did not induce a similar response.

Certain ecotypes of Pss, including that which is pathogenic towards bean (e.g., strain Y30), produce a phytotoxic peptide called syringomycin (SR) [21]. SR may be produced by Pss strain Y30 in soybean cell suspension cultures. The culture filtrate from the Pss strain Y30 and Sb-1 cell line interaction did induce chlorosis in bean leaves and slight growth inhibition of *G. candidum*. However, experimental

support for the presence of SR was not forthcoming when fresh soybean suspension cells were added to chlorosis-inducing culture filtrates since cell death did not occur. Also, under optimum conditions for SR production *in vitro* [22], very little SR was produced until Pss cultures reached the stationary phase. Usually, Pss strain Y30 caused the death of soybean suspension cells within 15 to 24 h, long before reaching stationary phase (approximately 72 h). Pss strain HVW3, although a higher toxin producer than strain Y30 when grown on PDA plus casamino acids, did not cause a similar precipitous decline in soybean cell viability. It would appear that Pss strain Y30 may be producing another phytotoxic substance not identical with SR or that strain Y30 is a better producer of SR than strain HVW3 under these cultural conditions.

We do not know if bacterial toxin production was responsible for the reduction of soybean suspension cell viability when challenged with Psg. Culture filtrates of the Psg strain A-29-2 and Sb-1 cell line interaction did cause slight chlorosis in leaves of bean. However, the slow reduction of soybean cell viability seen in most Psg-soybean suspension cell interactions over a 96 h incubation period might be due to competition between bacteria and host cells for available nutrients.

In this study, under the experimental conditions of soybean cell growth and bacterial challenge, we found that suspension cell cultures of cv. Mandarin did not mimic the response of whole plants to inoculation with *P. syringae* pathovars. In fact, there was no expression of compatibility or incompatibility to bacterial challenge as evidenced by the lack of differential glyceollin production, differential bacterial growth or an HR to incompatible *P. syringae* pv. *glycinea* and pv. *phaseolicola* strains. With further experimentation it is possible that race-specific responses of soybean suspension cells to Psg could be achieved. Race-specific resistance expression by cultured plant cells to *Phytophthora* sp. has been obtained after altering such factors as media hormone levels [26, 29], incubation temperature [28, 29], and callus thickness [29]. In addition, calluses derived from immune and susceptible cotton cultivars showed a differential reaction to two races of the cotton pathogen *X. campestris* pv. *malvacearum* (Smith) Dye [56]. Recently, an HR-like reaction of tobacco suspension cell cultures to *P. syringae* pv. *lisi* (Sackett) Young, Dye & Wilkie was reported [1].

Soybean cell suspension cultures may be of immediate use in studies of the mode of phytoalexin induction by phytopathogenic bacteria. Viable bacteria were capable of eliciting similar glyceollin production in soybean cell suspension cultures as *in vivo*. Also, as *in vivo* [35], heat-killed bacteria were not capable of eliciting glyceollin production. However, the problem of rapidly declining induced glyceollin production by soybean cell suspension cultures may necessitate frequent generation of cell suspension cultures from callus.

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