

## BACTERIALLY-INDUCED GLYCEOLLIN PRODUCTION IN SOYBEAN CELL SUSPENSION CULTURES

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### SUMMARY

Inoculation of suspension cultures of a recently initiated cell line of *Glycine max* (L.) Merr. cv Mandarin (Sb-2) with a compatible or incompatible strain of the soybean pathogen *Pseudomonas syringae* pv *glycinea* or the heterologous pathogen *P. syringae* pv *syringae* induced production of the phytoalexin glyceollin. Cell wall glucan from *Phytophthora megasperma* f. sp. *glycinea* was also an active elicitor. A cell line (Sb-1) of Mandarin suspension culture initiated in 1964 and subcultured ever since did not produce glyceollin in response to bacterial inoculation or treatment with various other biotic and abiotic elicitors. A bacterially-induced hypersensitive response was not required for glyceollin production.

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### INTRODUCTION

Plant tissue cultures have been used successfully for culturing biotrophic plant pathogens, for developing and maintaining pathogen-free plants, for generation of disease-resistant lines and in the study of plant-virus interactions [1]. However, plant tissue cultures have not been used extensively for the study of plant-fungal and especially plant-bacterial interactions [2]. Phytoalexins, induced plant constituents with antimicrobial activity [3], have been elicited in plant callus and cell suspension cultures by fungal spores or fungal cell wall elicitors [4] but notably not by bacterial cells or isolated components thereof.

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\*Agriculture Research Service, Science and Education, U.S. Department of Agriculture. Abbreviations: GC, gas chromatography; HR, hypersensitive response; MS, mass spectrometry; TLC, thin-layer chromatography.

This study was undertaken to determine if strains of *Pseudomonas syringae* pv *glycinea*, a leaf pathogen of soybean (*Glycine max* (L.) Merr.) [5], could induce a hypersensitive response and production of the pterocarpenoid phytoalexin glyceollin [6] by soybean cv. Mandarin cell suspension cultures.

## MATERIALS AND METHODS

### *Suspension cultures*

Two separate lines of cell suspension cultures of soybean cv. Mandarin were used. One (previously designated Sb-1) was initiated from root tissue in 1964 at the Prairie Regional Laboratory, Saskatoon, Sask. (Canada) [7] and had been subcultured ever since. A new line of Mandarin callus was obtained from surface sterilized epicotyl explants of seedlings on 1-B5 agar medium [7] supplemented with 0.15 mg kinetin l<sup>-1</sup>. Resulting calluses were transferred after 1 month to 2-B5 agar medium plus 0.15 mg kinetin l<sup>-1</sup>. After an additional month, the most friable calluses were placed in 1-B5 liquid medium and shaken to produce a cell suspension culture, designated Sb-2. All suspension cultures were maintained at 26–27°C on a rotary shaker at 150 oscillations min<sup>-1</sup> under constant illumination (9.84 lumens). Every 5–7 days approx. 20 ml of the culture was transferred to 40 ml of fresh 1-B5 medium in 250 ml flasks.

### *Bacterial cultures*

*Pseudomonas syringae* pv *glycinea* strain A-29-2 had been isolated from a Wisconsin soybean field by W.F. Fett and strain 1134 was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, U.K. *Pseudomonas syringae* pv *syringae* strain Y30, a pathogen of *Phaseolus vulgaris* L. but not of soybean, was obtained from the culture collection of the Department of Plant Pathology, University of Wisconsin, Madison, WI, U.S.A. All bacterial strains were maintained on King's medium B agar [8].

Pathogenicity of the *P. syringae* pv *glycinea* and *P. syringae* pv *syringae* strains were tested periodically by spray inoculating leaves of cv. Mandarin or *P. vulgaris* cv. Tenderwhite, respectively.

### *Other biotic and abiotic elicitors*

In addition to bacteria, the following biotic and abiotic elicitors were also tested: (a) purified mycelial cell wall glucan (primarily  $\beta$ -1,3 linked) derived from *Phytophthora infestans* and from *Phytophthora megasperma* f. sp. *glycinea*; (b) high (approx.  $7.5 \times 10^5$ ) and low (approx.  $3.0 \times 10^3$ ) molecular weight mycodextran (nigeran) preparations; (c) HgCl<sub>2</sub>.

The *P. infestans* cell wall glucan was obtained from S. Osman (this laboratory). Mycelia had been sonicated preceded by homogenization. The suspension was then autoclaved at 121°C for 30 min, centrifuged at  $48\,200 \times g$  for 60 min and the supernatant fluid collected and evaporated to dryness under vacuum. The residue was taken up in water (1 mg dry wt. ml<sup>-1</sup>) and 10 ml

was layered onto a column of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was eluted with 0.05 M phosphate buffer (pH 7.3) and the cell wall glucan fraction was collected after the void volume (80 ml) starting at 135 ml.

The *P. megasperma* f. sp. *glycinea* cell wall glucan was obtained from B. Valent and P. Albersheim. Mycelial cell walls had been subjected to partial acid hydrolysis followed by chromatography on a Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column. The void volume was collected and used without further purification.

#### *Glyceollin induction, isolation and quantification*

Cultures of Sb-1 and Sb-2 cells were used 2–4 days after transferring to fresh 1-B5 liquid medium. Cell suspensions of each cell line were pooled and then redistributed to 125-ml flasks at 25 ml flask<sup>-1</sup>. When testing for bacterially-induced glyceollin production the bacterial inoculum was prepared by suspending 24–48 h cells grown on King's medium B agar in sterile water, washing the cells 3 times and adjusting the bacterial concentration turbidimetrically. Bacterial inoculum (0.25 ml) was added to one flask per soybean cell line to give an approximate initial concentration of  $1 \times 10^7$  colony forming units ml<sup>-1</sup>.

Cell wall glucan from *P. infestans* in sterile water was added to the soybean cultures at 125 µg dry wt. ml<sup>-1</sup> culture while that from *P. megasperma* f. sp. *glycinea* was applied at 10 µg dry wt. ml<sup>-1</sup> culture. High and low molecular weight mycodextrans suspended in sterile water were added to the cell cultures at 100 µg dry wt. ml<sup>-1</sup> culture. HgCl<sub>2</sub> was added to the cell cultures to give a 10<sup>-3</sup> M concentration.

Soybean cell viability was followed by a dye exclusion test with 0.4% trypan blue [9].

After 24 or 48 h of incubation at 26–27°C, flask contents were lyophilized, ground to a fine powder and twice extracted with 70 ml EtOAc/MeOH (9 : 1) g<sup>-1</sup> dry wt. [10] by vigorous stirring for 5 min. The combined extracts were dried under a stream of N<sub>2</sub> and taken up in 1 ml 95% EtOH g<sup>-1</sup> dry wt. Fifty microlitres of each sample was used in a thin-layer chromatography (TLC) bioassay designed to detect activity against *Cladosporium* sp. [10]. The remaining sample was subjected to TLC on silica gel plates (250 µm) with fluorescent indicator (Analtech, Newark, DE, U.S.A) and developed in hexane/EtOAc/MeOH (60 : 40 : 1). Fluorescence-quenching areas corresponding to a glyceollin standard were scraped from the plates, packed into small glass columns and eluted with EtOH (9 ml). Eluates were dried under a stream of N<sub>2</sub> and the residue taken up in 2 ml EtOH. The UV spectrum of each sample was determined and glyceollin concentration calculated from the absorption at 285 nm and the molar extinction coefficient of 10 300 [11]. The presence of glyceollin in selected samples was further confirmed by mass spectra using a Hewlett-Packard 5992B GC/MS.

## RESULTS

*P. syringae* pv *glycinea* strain A-29-2 was pathogenic towards cv. Mandarin giving typical water-soaked leaf lesions within 4 days, while strain 1134 induced a typical hypersensitive response (HR) characterized by browning of the leaf laminae visible within 24–48 h [5]. *P. syringae* pv *syringae* strain Y30 was highly pathogenic toward cv. Tenderwhite giving typical water-soaked lesions within 4 days after inoculation [12]. By 1 week systemic chlorosis was evident on leaflets above the point of inoculation.

The presence of glyceollin in the cell suspension culture extracts was confirmed by fungitoxicity towards *Cladosporium* sp., behavior on TLC, UV spectra and GC-MS [10,13]. All 3 bacterial strains induced glyceollin production by Sb-2 (Table I). With both *P. syringae* pv *glycinea* strains, the amount of glyceollin increased from 24 to 48 h of incubation and the highest amount of the phytoalexin was elicited by strain A-29-2 at 48 h ( $237 \mu\text{g g}^{-1}$  dry wt.). Cultures inoculated with strain 1134 developed  $89 \mu\text{g g}^{-1}$  dry wt. at 48 h. The heterologous plant pathogen *P. syringae* pv *syringae* strain Y30 elicited glyceollin production but to a lesser level than the *P. syringae* pv *glycinea* strains with a decline from  $40 \mu\text{g g}^{-1}$  dry wt. at 24 h to  $16 \mu\text{g g}^{-1}$  dry wt. at 48 h. The only other elicitor, *P. megasperma* f. sp. *glycinea* cell wall glucan, tested against Sb-2 culture induced  $63 \mu\text{g glyceollin g}^{-1}$  dry wt. at 24 h.

TABLE I

GLYCEOLLIN CONTENT OF SOYBEAN CV. MANDARIN LINE SB-2 CELL SUSPENSIONS INOCULATED WITH FUNGAL CELL WALL ELICITOR OR *PSEUDOMONAS SYRINGAE* PVS *GLYCINEA* AND *SYRINGAE*

Treatment	Time of incubation (h)	Glyceollin ( $\mu\text{g/g}^{-1}$ dry wt)
<i>P. megasperma</i> f. sp. <i>glycinea</i> glucan <sup>a</sup>	24 <sup>b</sup>	63
<i>Pseudomonas syringae</i> pv <i>glycinea</i> A-29-2	24	32
	48	237
1134	24	32
	48	89
pv <i>syringae</i> Y30	24	40
	48	16

<sup>a</sup>Purified cell wall glucan ( $10 \mu\text{g dry wt. ml}^{-1}$  culture) from *Phytophthora megasperma* f. sp. *glycinea*.

<sup>b</sup>Values shown were obtained by subtracting control values from treatment values and are averages of data from 2 separate experiments.

Sb-1 cell cultures could not be induced to produce glyceollin by any of the three bacterial strains. Cell wall glucan from *P. infestans* and *P. megasperma* f. sp. *glycinea*, high and low molecular weight mycodextran, and HgCl<sub>2</sub>, also failed to elicit production of the phytoalexin by Sb-1. Both cell wall glucans were active glyceollin elicitors when used in a soybean cotyledon bioassay [11]. The *P. infestans* glucan-induced production of 1.5 μg glyceollin g<sup>-1</sup> fresh wt. μg<sup>-1</sup> dry wt. glucan and *P. megasperma* f. sp. *glycinea* glucan produced 2.0 μg glyceollin g<sup>-1</sup> fresh wt. μg<sup>-1</sup> dry wt. glucan.

Viable cells of both Sb-1 and Sb-2 at the start of each experiment ranged from 65% to 75%. Viability in control cultures remained fairly constant over 48 h of incubation. *P. syringae* pv *glycinea* strain A-29-2 caused a drop in viable Sb-1 cells to 40–50% after 48 h incubation. When incubation was extended to 72 h and 96 h, viability of Sb-1 was further decreased to 20–25% while no change in viability occurred in the control cultures up to 96 h. *P. syringae* pv *glycinea* strain 1134 had no effect on viability of Sb-1 up to 96 h.

Neither *P. syringae* pv *glycinea* strain affected viability of Sb-2 cells during 48 h incubation. The presence of *P. syringae* pv *syringae* strain Y30 caused a sharp decline in the percent viable Sb-1 and Sb-2 cells resulting in 3–12%, respectively, after 24 h and zero after 48 h. HgCl<sub>2</sub> caused complete killing of Sb-1 cells within 24 h. *P. megasperma* f. sp. *glycinea* cell wall glucan had no effect on viability of either Sb-1 or Sb-2 cells over 24 h. The effect of the mycodextrans and *P. infestans* cell wall glucan on cell viability was not determined.

#### DISCUSSION

We believe this to be the first report of phytoalexin elicitation in cultured plant cells by bacteria. In an extensive study, Kennedy et al. [14] failed to induce glyceollin production by soybean callus inoculated with *P. syringae* pv *glycinea* as well as several other bacteria.

The levels of glyceollin obtained in our study were lower than those previously reported for soybean tissue cultured cells exposed to various non-bacterial eliciting agents. Glyceollin induced by a *P. megasperma* f. sp. *glycinea* glucan (similar to that used in the present study) reached a maximum of 140 μg g<sup>-1</sup> fresh wt. soybean suspension cells [15] and UV irradiation produced as much as 836 μg g<sup>-1</sup> dry wt. soybean cells [16]. Keen and Horsch [17] successfully induced glyceollin formation by soybean callus (430–980 μg g<sup>-1</sup> dry wt.) on inoculation with zoospores of *P. megasperma* f. sp. *glycinea*, but found no differences in this regard between callus derived from susceptible or resistant soybean cultivars.

No HR, characterized by the rapid death of host cells [18], occurred after inoculation of the incompatible strain (1134) of *P. syringae* pv *glycinea*. However, *P. syringae* pv *syringae* strain Y30 did cause rapid death of both Sb-1 and Sb-2 cell lines. Rapid death of the host cells did not permit in-

creased accumulation of glyceollin with time; in fact, the decline in glyceollin from 24 h to 48 h suggests its degradation by strain Y30. We are currently examining this rapid host cell death to ascertain if it was an HR or resulted from the production of bacterial toxin(s). Host inoculation demonstrated strain Y30 to be a potent toxin producer in vivo.

The results of the interaction between strains of *P. syringae* pv *glycinea* and soybean cell suspension cultures did not fully mimic in vivo phenomena [19]. No HR occurred and higher amounts of glyceollin were found in the compatible interaction. However, the results with suspension cultures do indicate that the bacterially-induced HR in vivo is apparently not a prerequisite for glyceollin production. Also, cell lines Sb-1 (in our laboratory) and Sb-2 appear to differ in their ability for induced glyceollin production and might be useful as an experimental tool to study phytoalexin induction, biosynthesis and regulation.

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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.

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