Metabolites from *Pseudomonas corrugata* Elicit Phytoalexin Biosynthesis in White Clover

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Contribution 8812 of the U.S. Regional Pasture Research Laboratory, University Park, PA.

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We gratefully acknowledge the technical assistance of R. G. Pedersen. We thank Nicole R. O'Neill, Kenneth T. Leath, and Eva J. Pell in their helpful suggestions in preparation of the manuscript.

Accepted for publication 6 July 1990 (submitted for electronic processing).

ABSTRACT

Gustine, D. L., Sherwood, R. T., Moyer, B. G., and Lukezic, F. L. 1990. Metabolites from *Pseudomonas corrugata* elicit phytoalexin biosynthesis in white clover. Phytopathology 80:1427-1432.

Metabolites and viable cells of *Pseudomonas corrugata* from liquid culture medium elicited biosynthesis of the phytoalexin medicarpin in ladino white clover (*Trifolium repens*) leaflets and callus. The biologically active elicitor components were soluble in 80% ethanol. They were partially purified by removing components greater than 3,500 Da by dialysis and fractionating by preparative reversed-phase high-performance liquid chromatography (HPLC). None of the four fractions separated by HPLC elicited appreciable quantities of medicarpin in callus, but fraction 1 combined with fraction 4 elicited high concentrations of medicarpin. Any combination of fractions 2, 3, and 4 synergistically elicited medicarpin in callus. Elicitor activity was concentration-dependent. The active fractions were acidic in solution, but their elicitor activity was not dependent on low pH. Fraction 1 contained primarily uncharacterized reducing carbohydrate and phosphate. Fractions 2 and 3 were composed primarily of two related, unidentified fluorescent compounds, and fraction 4 contained another unidentified fluorescent compound.

Additional keywords: bacterial elicitor, synergism, tissue culture.

The pseudomonads are a major group of plant pathogens that cause necrotic leaf spots, vascular wilt, and soft rot diseases in agriculturally important crops. *Pseudomonas corrugata*, described by Scarlett et al (18), is a pathogen of greenhouse-grown tomato plants. The strain isolated by Lukezic (17) from symptomless alfalfa roots caused localized necrosis in alfalfa stems, rot in onion scales, and necrotic lesions in lettuce leaves (17). Live cells of *P. corrugata* elicit the phytoalexin medicarpin in ladino white clover callus tissue cultures (11).

The biosynthesis and accumulation of phytoalexins in affected host cells at concentrations inhibitory to the pathogen may prevent further development of infection (4). The phytoalexin response is thought to be caused by metabolites (elicitors) that interact with a specific component of the host cell (4,9). Although elicitors have been characterized from many fungal pathogens (3,4), none have been characterized from bacteria.

An experimental system well suited to testing phytoalexin elicitor activity of microorganisms or extracts and fractions from them is the induction of medicarpin biosynthesis in ladino clover callus (11). Using this bioassay system, we followed the partial purification of phytoalexin elicitors from *P. corrugata*. We demonstrate here that this plant pathogen produced metabolites in culture medium that induce phytoalexin accumulation in ladino white clover leaflets and tissue culture. Partially purified components acted synergistically and induced higher than expected phytoalexin accumulation. A preliminary report of this work has been published (13).

MATERIALS AND METHODS

Bacterial culture. A strain of *P. corrugata* (RB15) obtained from roots of alfalfa (17) was used; it retained its virulence on alfalfa and tomato during the course of experiments. For long-term storage, bacteria were cultured on YDCA (22), suspended in powdered milk solution, and lyophilized. For short-term storage at room temperature, cells were maintained as stabs in 1% water agar. Inoculum for liquid cultures was prepared by suspending bacteria from 24-hr cultures grown at 24 C on petri plates containing YDPA (yeast extract, 3 g; glucose, 20 g; phytopeptone, 5 g; and agar, 15 g/L) in 10 ml of water. Liquid cultures (0.1 ml of inoculum) were grown in 250 ml of double-strength basal inorganic medium B (8) with 0.01 g of $Fe_2(SO_4)_3$ per liter and supplemented with 0.4% glucose. Liquid cultures were incubated for 24 hr at 24 C on an orbital shaker.

Preparation of elicitor fractions. Liquid culture (2 L) of *P. corrugata* was processed as shown in Figure 1. Following centrifugation of the culture, the supernatant fraction was lyophilized (prep 1b). For initial experiments, the residue was dissolved in 200 ml of water and mixed with 670 ml of absolute ethanol (procedure 1); later, and for most of the experiments, the amount of ethanol was arbitrarily increased to 800 ml (procedure 2) to ensure maximum precipitation of medium components. After 1 hr at 4 C, a white precipitate was removed by centrifugation and dissolved in 20 ml of H_2O (prep 2a). To remove ethanol, the supernatant fraction was reduced in volume to 50 ml by flash evaporation at 34 C (prep 2b). This fraction was placed in Spectrapor dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) with a molecular weight cutoff of 14,000 (procedure 1) or 3,500 Da (procedure 2) and dialyzed twice for 3 hr at 4 C in 500 ml of water. The combined dialysates were divided into five equal portions and lyophilized (prep 3b). Freeze-dried dialysates were stored at −20 C up to 6 mo without loss of activity. Although the procedures for preparing the dialysate fraction (prep 3b) varied slightly, dialysate activity in the callus assay relative to $HgCl_2$ control treatment was reproducible. To check for nonspecific elicitor activity of the growth medium, 1 L of autoclaved, uninoculated medium was fractionated by procedure 2.

One-fifth of the dialysate (prep 3b) from procedure 2 was reconstituted in 4 ml of water and further purified by preparative-phase HPLC. This dialysate was filtered through a Gelman Acrodisc-LC13 (0.45-μm cutoff) (Gelman Sciences, Inc., Ann Arbor, MI) and injected. Preparative reversed-phase HPLC was performed with a 0.7- × 50-cm stainless steel column packed with Waters reversed-phase packing (55–100 μm) (Water Associates, Milford, MA), equilibrated with 1% methanol and 2 mM acetic acid in water. Flow rate was 7 ml/min. Effluent was monitored simultaneously with a Waters 440 UV detector (254 nm) and a Waters 420 refractive index (RI) detector coupled with a dual pen recorder. Three fractions from each injection were collected manually via the collection port on a Waters 6000A pump; usually, 12 injections were sufficient to process 4 ml of prep 3b. Each of the three fractions from 12 injections was collected and pooled. A fourth fraction was collected by decreasing the solvent polarity to 70% methanol and eluting strongly bound components that had accumulated from the multiple injections. The four fractions were lyophilized and stored at −20 C until further testing.

Phytoalexin induction. Callus cultures of ladino white clover (*Trifolium repens* L.) were initiated and maintained as described (10,12). Bioassay incubations consisted of 0.5 ml of double-strength modified Gamborg's B5 medium (10) and 0.5 ml of test

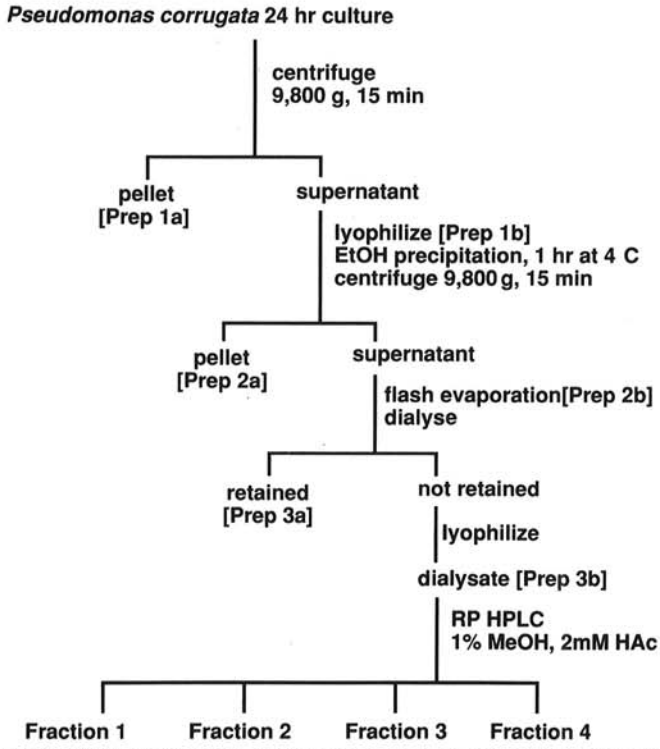

Fig. 1. Elicitor fractionation scheme. In procedure 1, ethanol concentration was 67%, and the dialysis membrane had a molecular weight cutoff of 14,000 Da. In procedure 2, ethanol concentration was 80%, and the dialysis membrane had a molecular weight cutoff of 3,500 Da. RP: reversed phase; EtOH: ethanol; MeOH: methanol.

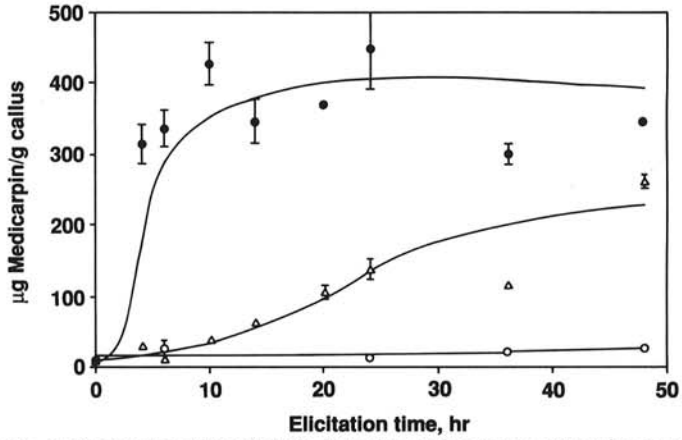

Fig. 2. Production of medicarpin in ladino white clover callus bioassay system induced by *Pseudomonas corrugata* at 3×10^8 cells/ml (Δ–Δ) and 1 mM $HgCl_2$ (●–●); water control (○–○). Incubation vials for each time point were prepared as described in Materials and Methods and prepared callus was added at the same time to each vial. Five minutes before the end of a time period, callus was filtered, divided into three replicate portions, and the biosynthesis stopped by storing the tissue at −70 C. Tissue was thawed during extraction of medicarpin with 2-butanone, and medicarpin analyzed. Vertical bars indicate standard deviation of greater than ± 6 μg of medicarpin per gram of callus.

fluid (suspension of *P. corrugata* at 5×10^8 cells per milliliter of water, solutions of preparations, or solutions of HPLC fractions) and 1.5–2 g of callus prepared as in (10) in 7-ml plastic weighing vials. Callus was added and mixed with the test fluid to initiate elicitation and incubated for 24 hr at 24 C. Medicarpin was extracted from three weighed replicates per vial of filtered callus and analyzed by normal-phase HPLC (10). All bioassays included 0- and 24-hr untreated controls. Because callus preparations exhibited varied response to elicitors, a treatment with 1 mM $HgCl_2$ was included in each set of bioassays as a positive control.

Phytoalexin accumulation in ladino white clover leaflets was determined by the drop-diffusate method (15). Detached leaflets (three replicates, 30 leaflets per replicate) were placed on wet Whatman 3MM filter paper. Dialysate (prep 3b, procedure 2), 20× more concentrated than the starting culture fluid, or cells of *P. corrugata* suspended in water (5×10^8 bacterial cells per milliliter) was placed on leaflets as drops (70 μl) and incubated under high humidity for 24 hr. The positive control was 1 mM $HgCl_2$ and the baseline control was water. All treatments contained 0.1% Tween 20. Drops were pooled from each treatment for each replicate and medicarpin partitioned into ethyl acetate (three times, 3.5 ml). Leaflets were extracted by grinding with a mortar and pestle in 70% methanol (20 ml/g of tissue). After evaporation of the extracts to dryness in vacuo, the residues were extracted with ethyl acetate-hexane (20:80, v/v) and medicarpin concentration determined by HPLC (10).

Analytical procedures. Reducing carbohydrate was determined as glucose by the procedure of Hodge and Hofreiter (14); protein was determined as bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) by the procedure of Flores (7); phosphate was determined as NaH_2PO_4 by the procedure of Ames (2). Ultraviolet spectra of preparative HPLC fractions in water were obtained with a Gilford Model 2400S spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Analytical reversed-phase HPLC was performed with a 0.46- × 25-cm Supelco LC18 column (Supelco, Inc., Bellefonte, PA) in 1% methanol and 2 mM acetic acid at a flow rate of 0.8 ml/min. TLC analyses of elicitor-active fractions were performed with Merck-SiGel 60 0.25-mm thick plates (Merck & Co., Inc., West Point, PA) developed in 1-butanol/glacial acetic acid/water (4:1:1, v/v/v). Deionized water (17 Mohm) was used for all analytical procedures. All solvents for reversed-phase and normal-phase HPLC were filtered (0.45 μm).

RESULTS

Phytoalexin induction. The biosynthesis and accumulation of medicarpin in elicitor-treated ladino clover callus is well characterized (11). This system was adapted to provide a bioassay to measure elicitor activity of *P. corrugata* and fractions from bacterial culture fluid. Nonelicited concentrations of medicarpin were 25–50 μg/g fresh wt of callus over a 24-hr incubation period (Fig. 2 and Tables 1–3). Callus elicited with 1 mM $HgCl_2$ or *P. corrugata* produced up to 600 μg of medicarpin per gram (Fig. 2, Tables 1–3). Medicarpin increased linearly from 3 to 8 hr (10), following addition of 1 mM $HgCl_2$ and from 10 to 24 hr following addition of 5×10^8 cells of *P. corrugata* per milliliter, and remained at maximum level through 48 hr (Fig. 2). The concentration of medicarpin at 24 hr was dose-dependent for $HgCl_2$ (10) and *P. corrugata* (Table 1). No symptoms of necrosis were observed in callus after 48 hr of incubation with *P. corrugata* or any fractions. Callus tissue darkened slightly after a 24-hr incubation in 1 mM $HgCl_2$. Slight browning of white clover leaflets was observed under droplets of *P. corrugata* and prep 3b, and leaflet tissue under $HgCl_2$ appeared necrotic.

To show that elicitation of medicarpin in callus was not an artifact of culture, white clover leaflets were challenged with 1 mM $HgCl_2$ or 5×10^8 cells of *P. corrugata* per milliliter. Medicarpin levels in white clover leaflets and diffusates were lower than found in callus. Medicarpin concentrations (± SD, $n = 3$) were 51.1 ± 9.3 and 15.4 ± 2.6 μg/ml of diffusate from $HgCl_2$ or treatments of *P. corrugata*, respectively, and 39.5 ± 9.4 and 17.3 ± 2.4 μg/g fresh wt of leaflets from $HgCl_2$ or treatments of *P. corrugata*, respectively. Medicarpin increased 5- to 15-fold over nonelicited levels in response to *P. corrugata* or $HgCl_2$ in the callus system and 7- to 17-fold in the leaflet system. Treatment with dialysate (prep 3b) produced medicarpin at 23.4 ± 0.7 μg/g fresh wt (leaf + diffusate), a fourfold increase over the control. This result was obtained in three experiments.

TABLE 1. Response of ladino white clover callus to cell density of *Pseudomonas corrugata*

Cells/ml of assay fluid ($\times 10^{-8}$)	Medicarpin concentration[a] (μg/g fresh wt ± SD)
0	34.6 ± 2.6
0.1	25.6 ± 4.8
0.2	36.3 ± 8.6
0.5	61.9 ± 7.9
1.0	73.7 ± 16.1
2.0	95.9 ± 0.9
5.0	188.2 ± 20.7
$HgCl_2$(1 mM)	561.9 ± 19.9

[a] Medicarpin concentrations are expressed as the mean of three determinations, ± standard deviation. Bioassays were conducted with a single batch of callus prepared from 27 2-g pieces of ladino white clover callus.

TABLE 2. Elicitor activity of fractions from culture fluids of *Pseudomonas corrugata*[a]

Fraction	Equivalent concentration[b]	Medicarpin concentration (μg/g fresh wt ± SD[c])
Prep 1b	6×	478.0 ± 64.0
Prep 2a	6×	174.3 ± 40.5
Prep 2b	6×	525.9 ± 78.1
Prep 3a	10×	250.6 ± 33.4
Prep 3b	10×	447.9 ± 56.5
$HgCl_2$ control (1 mM)	...	626.3 ± 60.1
Water control	0×	42.6 ± 7.8

[a] Bacteria were inoculated into culture medium (2 L), grown for 24 hr, and the culture fluid centrifuged at 9,800 *g* for 15 min. The volume of the supernatant was reduced to 200 ml by flash evaporation and beginning with ethanol precipitation, was fractionated by procedure 1 as described in Materials and Methods. Bioassays were conducted with a single batch of callus prepared from 24 2-g pieces of ladino white clover callus.
[b] Increase in concentration relative to the starting culture fluid.
[c] SD = standard deviation; $n = 3$.

TABLE 3. Elicitor activity of dialysates from autoclaved, uninoculated culture medium and culture fluid of *Pseudomonas corrugata*[a]

Fraction	Equivalent concentration[b]	Medicarpin concentration (μg/g fresh wt ± SD[c])
Prep 3b (*P. corrugata* culture)	10×	206.6 ± 5.1
	4×	102.4 ± 8.4
	1×	81.5 ± 10.8
Prep 3b (Uninoculated medium)	10×	55.9 ± 16.8
	4×	35.9 ± 5.8
	1×	38.0 ± 3.5
$HcCl_2$ (1 mM)	...	364.0 ± 13.5
Water control	0×	39.0 ± 6.0

[a] Bacteria were inoculated into culture medium (2 L), grown for 24 hr, and the culture fluid fractionated by procedure 2 as described in Materials and Methods. Bioassays were conducted with a single batch of callus prepared from 24 2-g pieces of ladino white clover callus.
[b] Increase in concentration relative to the starting culture fluid.
[c] SD = standard deviation; $n = 3$.

Elicitor purification. Two liters of 24-hr bacterial culture was produced and elicitor activity fractionated by procedure 1 as outlined in Figure 1. After centrifugation of the culture medium, the culture supernatant (prep 1b, sixfold more concentrated than the culture fluid) elicited medicarpin biosynthesis in the callus bioassay (Table 2), as did 5×10^8 bacterial cells per milliliter (prep 1a, data not shown). Active components were apparently produced in low concentrations, since it was necessary to concentrate the cellfree culture medium to detect activity equivalent to 5×10^8 of suspended bacteria cells per milliliter. No attempt was made to isolate active fractions from the bacterial pellet. After addition of ethanol to 10× culture fluid, more elicitor activity was found in the soluble fraction (prep 2b) than in the precipitate (prep 2a) (Table 2). After dialysis, more elicitor activity was found in prep 3b than in prep 3a (Table 2), indicating the bulk of the elicitor-active components was smaller than 14,000 Da. The elicitor activity of prep 3b tested at 10× was equivalent to that of the original culture fluid (prep 1b) tested at 6× (Table 2). Because more extensive dialysis did not increase elicitor activity in the low molecular weight fraction (data not shown), we only dialyzed twice against 500 ml of water to minimize the time and volume for this step.

To estimate the size range of elicitor-active metabolites of *P. corrugata*, 2 L of culture was grown and elicitor activity fractionated by procedure 2 (see Fig. 1). Although elicitor response of the callus reported in Table 3 was low, prep 3b, obtained from dialysis through a 3,500-Da cutoff membrane, was active in the callus bioassay. Furthermore, the elicitor activity of the fraction was dosage-dependent (Table 3). Elicitor activity was not an artifact of the procedure or of the medium, since no significant activity was observed in prep 3b prepared from uninoculated, autoclaved medium (Table 3). Preps 1a through 3a obtained by procedure 2 were not tested in the callus assay, because activity of the dialysate (prep 3b) obtained by procedure 2 was consistently as high as dialysate obtained by procedure 1 (data not shown).

Dialysate preparations from either procedure were strongly acidic, having a pH in solution of 3.0–3.8. However, low pH itself was not responsible for induction of medicarpin biosynthesis. When a portion of the dialysate (prep 3b, procedure 2) was adjusted from pH 3.3 to 6.4 with 1 N NaOH and tested at 14× in callus, medicarpin concentrations at 24 hr were 240.5 ± 33.5 and 246.4 ± 25.6 μg/g callus for pH 3.3 and 6.4, respectively (see Table 3 for controls).

Because chromatography of the dialysate (prep 3b) on Sephadex G-25 or G-50 columns did not further fractionate elicitor activity (data not shown), preparative reversed-phase HPLC was performed. A single peak of unretained material (fraction 1) eluting at 1.2 min was observed with the RI detector and two partially retained peaks (fractions 2 and 3) eluting at 4.8 and 10 min were observed with the UV detector. To process 4 ml of dialysate, a series of injections was made and each of the three fractions was collected and pooled. After changing the mobile phase to 70% methanol, a UV peak of less polar components was collected (fraction 4). Five dialysate preparations from three separate cultures of *P. corrugata* were fractionated by reversed-phase preparative HPLC. The amount of material recovered in the four fractions ranged from 98 to 102% of the starting dialysate, on a dry weight basis. The results from two of the chromatographic separations are shown in Table 4. The fractions did not contain protein, but fraction 1 contained 53 and 54% reducing carbohydrate in the two preparative separations, with similar percentages in the three other separations. Low absorbance values were found for fractions 1 and 4, but fractions 2 and 3 in solution were yellow in color with maximum absorption around 250 and 400 nm (Table 4). Either production of fractions 2 and 3 was variable, or quantitative recovery of the fractions varied (see absorbance data, Table 4). Three other dialysate preparations fractionated by reversed-phase HPLC yielded essentially identical separations and similar data. Data from HPLC separation II show that fraction 1 contained reducing carbohydrate and inorganic phosphate (Table 4). The carbohydrate and phosphate components accounted for approximately 85% of the material in fraction 1. The composition of the remaining 15% of fraction 1 is unknown. The spectrophotometer readings for colorimetric analysis of fractions 2–4 were not significantly different from reagent blanks. Thus, these fractions were essentially free of reducing carbohydrate and phosphate. The measured pH of the four fractions ranged from 3.2 to 5.8, but the chemical group(s) responsible for their acidic nature is not known. TLC analysis indicated that fraction 2 was composed primarily of one compound having an R_f of 0.3. The compound had a faint yellow color under visible light, but under long UV light was light yellow and fluorescent. Fraction 3 was composed primarily of one compound at R_f 0.6. This compound had a faint, bright yellow color under visible light and a yellow-green fluorescent color under UV light. Fraction 4 contained a major component at R_f 0.8, that was faintly yellow under visible light and was deep violet under UV light. Fraction 1 contained traces of the R_f 0.3 and 0.6 compounds, fraction 2 contained some R_f 0.6 compound, and fraction 3 contained some R_f 0.3 compound. The TLC data suggest that the R_f 0.3 and 0.6 compounds are closely related and exist in an equilibrium. This accounts for the trace amounts of both compounds in fraction 1 as a result of incomplete separation of fractions 1 and 2.

Synergistic elicitor activity. The activity of fraction 1 was about 50% lower than the dialysate activity (Fig. 3). When fractions 2–4 were bioassayed in callus at concentrations 20× more concentrated than the starting culture fluid, elicitor activities were

TABLE 4. Properties of fractions from reversed-phase high-performance liquid chromatography (HPLC) of dialysates[a] from *Pseudomonas corrugata*

Fraction	Weight (mg)	pH	Reducing carbohydrates (mg)	Phosphate (mg)	A (λmax in nm)
HPLC Separation I					
1	960.0	3.2	506.7	ND[b]	ND
2	13.5	3.8	4.6	ND	18.8(252), 9.3(408)
3	3.6	4.6	1.3	ND	52.2(248), 19.2(398)
4	2.0	4.3	0.2	ND	ND
HPLC Separation II					
1	757.0	3.8	410.0	230.0	ND
2	8.0	5.2	2.0	0.8	8.6(250), 5.5(407)
3	16.0	2.8	1.7	0.5	10.1(249), 5.3(400)
4	1.0	5.8	0.2	0.2	ND

[a] Dialysates (prep 3b, procedure 2) were prepared from two separate 2-L cultures of *P. corrugata*.
[b] Not determined.

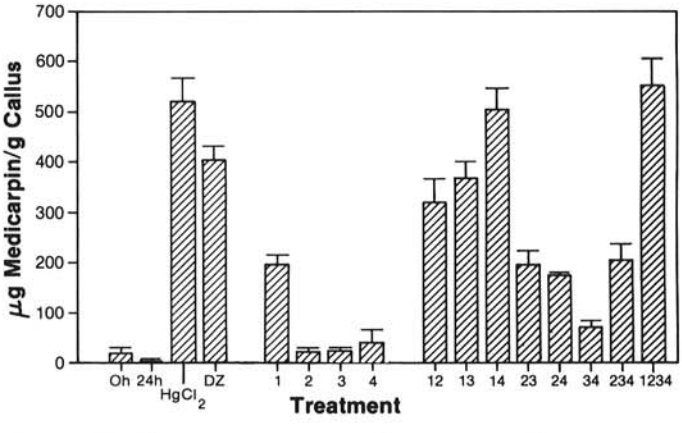

Fig. 3. Callus bioassay response to individual and combined reversed-phase HPLC fractions 1 through 4. Fractions were tested at a concentration of 20× relative to original culture fluid. Actual amounts tested per assay were: fraction 1, 49.9 mg; fraction 2, 0.74 mg; fraction 3, 0.04 mg; fraction 4, 0.2 mg. Each treatment was corrected for 24-hr water control. DZ = dialysate; 12,13...1234 indicate combinations of fractions 1, 2, 3, or 4. Vertical bars indicate standard deviation.

not significantly different from 0- or 24-hr controls (Fig. 3). Combinations of fractions were tested to determine if elicitor activity had been lost or now required a coelicitor. Two of eight combinations produced bioassay responses greater than that for the dialysate (1 + 4 and 1 + 2 + 3 + 4 in Fig. 3), and two produced responses >80% of the dialysate (1 + 2 and 1 + 3). Combinations that did not include fraction 1 produced responses <50% of the dialysate, but all combinations except 3 + 4 produced a greater response than the sum of the responses to individual fractions. Combinations containing fraction 4 and fraction 1 elicited the highest levels of medicarpin biosynthesis in callus. Both fractions 1 and 4 showed a concentration dependence for the other fraction (Fig. 4) in eliciting the response.

DISCUSSION

P. corrugata causes a spreading necrotic lesion up to 100 mm long when inoculated into a compatible host, tomato (18), and up to 5 mm long when inoculated into an incompatible host, alfalfa (17). *P. corrugata* also causes a typical hypersensitive response in 24 hr when infiltrated at a concentration of 5×10^8 bacterial cells per milliliter into alfalfa and ladino white clover leaflets (data not shown). Thus, the ladino white clover/*P. corrugata* incompatible system appears promising for studying elicitation of resistance in this forage legume. This was confirmed by the demonstration that inoculation of leaflets or callus induced accumulation of the phytoalexin medicarpin within 24 hr.

One of our objectives was to find a cellfree product of the bacterium that could be used for studying membrane-associated interactions at the molecular level that lead to elicitation of phytoalexins. We have established that cellfree culture fluid of *P. corrugata* contained metabolites capable of eliciting phytoalexin biosynthesis in ladino white clover callus (Table 2).

Dialysate could be separated by preparative reversed-phase HPLC into four fractions, of which only fraction 1 retained partial elicitor activity. When these fractions were combined and tested in callus, maximum level of phytoalexin concentration was restored. Relatively high amounts (50–60 mg/ml) of fraction 1 were needed for elicitation as indicated for Figures 3 and 4. This raises the question whether the primary effect of fraction 1 was due to osmotic or ionic effects, or a minor constituent of fraction 1. Fractions 2, 3, and 4 showed little or no difference from water controls when tested singly (Fig. 3). Combinations of two or all three fractions resulted in synergistic interaction and produced significant elicitor activity. The addition of fractions 2, 3, or 4 to fraction 1 gave elevated activity over fraction 1 alone, further indicating synergistic action. When used in combination with each other or with fraction 1, fractions 2, 3, or 4 gave synergistic activity at total concentrations in the microgram range, concentration levels approaching those reported for cell wall-derived fungal elicitors (4). Davis et al (5) previously reported that abiotic elicitors combined with endogenous host cell wall-derived elicitors acted synergistically in soybean. In contrast, our results demonstrate synergism of the exogenously added pathogen metabolites, without a requirement for production of host-derived coelicitors.

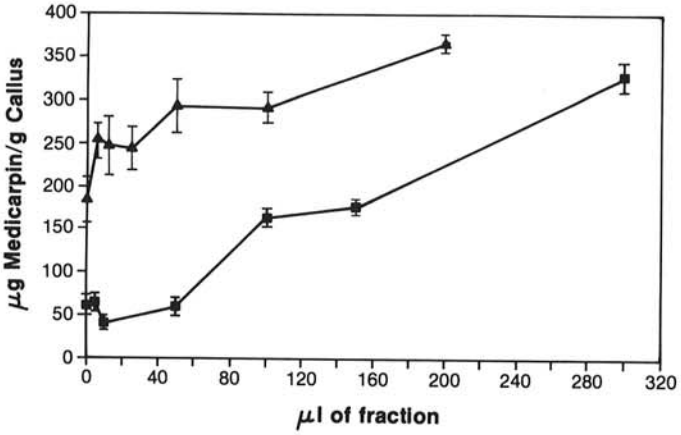

Fig. 4. Dosage-response of medicarpin levels in induced callus to combinations of fractions 1 and 4. Legend: ▲–▲ 62.5 mg of fraction 1 (15× concentrated relative to original culture fluid) and different amounts of fraction 4 at 0.0043 mg/ml; ■–■ 0.064 mg fraction 4 (15× concentrated relative to original culture fluid) and different amounts of fraction 1 at 0.25 mg/ml. Each treatment was corrected for 24-hr water controls. $HgCl_2$ controls were 659.3 and 731.0 μg of medicarpin per gram for the top and bottom curves, respectively. Vertical bars indicate standard deviation.

Reports of bacterial elicitation of phytoalexins are rare. Moreover, bacterial elicitors have not been characterized. Albersheim and Wolpert (1) suggested that cells or lipopolysaccharides of *Escherichia coli* can stimulate phytoalexin production in soybean cotyledons. Glyceollin I accumulation was observed in inoculated ineffective soybean nodules in which *Rhizobium japonicum* peribacteriod membrane failed to form (21). In the case of effective symbiosis, glyceollin was not detected in nodule tissue. More recently, Keen et al (16) reported an uncharacterized extracellular elicitor of HR produced by transformed *E. coli* containing the *arvD* gene from *P. syringae* pv. *tomato*. Some cultivars of soybean treated with the elicitors contained glyceollin at quantities up to 500 μg/g fresh wt of leaves.

Many elicitors described in the literature are polysaccharide fragments derived from fungal cell walls, but may also be products of host cell wall biodegradation (3,4). Endopolygalacturonase from *Erwinia carotovora* was an efficient elicitor in soybean because it released elicitor-active 1,4-linked oligogalacturonide fragments from the host cell wall (6). Thus, although interactions of *E. carotovora* with the host resulted in phytoalexin accumulation, this bacterium does not itself produce an elicitor. Because the active fractions from *P. corrugata* are <3,500 Da, polygalacturonases or β-glucanases acting on the host cell wall to produce elicitors can be ruled out.

The elicitor active fractions from *P. corrugata* are unique because they are pathogen derived, they do not require host cofactors, and they act synergistically. Purification and identification of their chemical structures is required to understand their possible function in elicition of phytoalexin accumulation. With these elicitors it may be possible to locate and characterize specific host-plant membrane receptors (10,19,20,23) that function in controlling activation of phytoalexin biosynthesis.

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