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Host-Pathogen Interactions

X. FRACTIONATION AND BIOLOGICAL ACTIVITY OF AN ELICITOR ISOLATED FROM THE MYCELIAL WALLS OF *PHYTOPHTHORA MEGASPERMA* VAR. *SOJAE*[1]

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ABSTRACT

An elicitor of phytoalexin production in soybean (*Glycine max* L.) tissues was isolated from purified *Phytophthora megasperma* var. *sojae* mycelial walls by a heat treatment similar to that used to solubilize the surface antigens from the cell walls of *Saccharomyces cerevisiae*. The wall-released elicitor is a discrete, minor portion of the *P. megasperma* var. *sojae* mycelial walls. The elicitor released from the mycelial walls was divided by diethylaminoethylcellulose and concanavalin A-Sepharose chromatography into four fractions, each having different chemical characteristics. The four fractions were obtained from each of the three races of *P. megasperma* var. *sojae*. The corresponding fractions from each of the three races are very similar in composition and elicitor activity. The results suggest that the elicitor activity of each fraction resides in the glucan component of the fraction. Evidence is presented to demonstrate that the elicitors are not race-specific and that the accumulation of glyceollin is not sufficient to account for race-specific resistance.

The purification and characterization of the elicitor present in the extracellular medium of Pms[5] cultures was described in the previous paper (3). This extracellular elicitor was demonstrated to be a polysaccharide with a composition and structure similar to that of the base-insoluble glucan of the mycelial walls of Pms (17). The similarity between the extracellular elicitor and this wall polysaccharide suggests that the extracellular elicitor is originally an element of the mycelial walls and that the elicitor is released into the medium of aging Pms cultures. The results (3) provide the first instance in which a pathogen wall component has been implicated in plant disease resistance. These observations led to the experiments described in this paper in which the elicitor was isolated from purified mycelial walls of Pms.

The three races of Pms are distinguished by different abilities to infect various soybean cultivars. A Pms-soybean combination which supports the growth of a specific race of Pms is termed a compatible interaction, and, conversely, an interaction is incompatible if the growth of the pathogen is restricted. The cultivar of soybean used in this study is Harosoy 63. This cultivar is compatible with race 3 of Pms, but incompatible with races 1 and 2.

It has been suggested (10, 12, 13) that glyceollin accumulates more rapidly in an incompatible Pms-soybean interaction than in a compatible interaction. This rapid accumulation is thought to be responsible for the resistance of soybeans to incompatible races of Pms. Elicitors from incompatible races of Pms would be expected to stimulate glyceollin accumulation, whereas corresponding molecules isolated from compatible races of Pms should not stimulate glyceollin accumulation. The experiments described in this paper provide measurements of the relative abilities of the elicitors from the three races of Pms to stimulate glyceollin synthesis in Harosoy 63 tissues. These measurements permit a new assessment of the role of phytoalexins in disease resistance. Data are presented which indicate that elicitors are not the primary determinants of race-specific resistance in soybeans infected by Pms.

MATERIALS AND METHODS

The cotyledon, hypocotyl, and suspension culture assays for elicitor activity have been described (3, 8).

Combined Use of Anthrone and Alditol Acetate Methods for Determination of Carbohydrate Content of Elicitor Fractions. Different sugars produce a different specific absorbance in the anthrone assay (7). Glucose and mannose, the primary constituents of the Pms elicitor, give quite different responses. One hundred μg of glucose results in an A_{620} of 1.40 in the anthrone assay while 100 μg of mannose results in an A_{620} of only 0.70. Amounts of elicitor have been expressed in the previous paper (3) as the μg equivalents of glucose required to give the same A_{620} reading as the elicitor preparation. These values are essentially correct for the extracellular elicitor, because this elicitor is composed almost entirely of glucose. These values would, however, be underestimates of the actual amounts of mannose. The amount of carbohydrate in an elicitor fraction as determined by the anthrone method is corrected in this and the following paper (4) for the amount of mannose present in each fraction.

The ratio of glucose to mannose in each preparation was determined by the alditol acetate assay (9). The ratio of glucose to mannose for the four wall-released elicitor fractions is: I, 19; II, 0.16; III, 0.48; and IV, 0.20. The correction factor for each fraction was determined by the equation

$$f = \frac{R + 1}{R + x}$$

where f is the correction factor, R is the ratio of glucose to mannose, and x is the ratio (0.5) of the A_{620} values of mannose and glucose in the anthrone assay. The correction factors (f) for the four fractions are: I, 1.03; II, 1.76; III, 1.51; and IV, 1.71. The mannose content of each fraction is accounted for by multi-

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[5] Abbreviations: Pms: *Phytophthora megasperma* var. *sojae*.

plying the anthrone-determined glucose equivalents of a fraction by the computed correction (*f*) factor for that fraction.

Purification of Pms Mycelial Walls. The culture conditions for Pms have been described (3). One hundred g batches of mycelia were harvested by suction filtration on a coarse sintered-glass funnel from liquid cultures of Pms races 1, 2, and 3. All subsequent steps were performed at 4 C.

The mycelia were washed on a coarse sintered-glass funnel with 4 liters each of distilled H_2O, of 100 mM potassium phosphate, pH 7.2, and of 500 mM potassium phosphate, pH 7.2. The mycelia were then homogenized in a Waring Blendor in 250 ml of the 500 mM buffer. The homogenized mycelia were washed 8 times with 500-ml aliquots of the 500 mM buffer by suction filtration through Nitex nylon 37-μm opening monofilament screen (No. 3-400-37 cloth, Tetko, Inc., N. Y.) supported on a coarse sintered-glass funnel. The remaining cell membrane and wall material was washed with 8 liters of deionized H_2O and 2 liters of chloroform-methanol (1:1, v/v). The mycelial walls were then washed with 1 liter of acetone and were air-dried.

Elemental Analysis of Purified Mycelial Walls of Pms. The carbon, hydrogen, oxygen, nitrogen, ash, and water content of purified mycelial walls were determined by Huffman Laboratories, Denver, Colo.

RESULTS

Composition of Purified Mycelial Walls of Pms. The elemental analysis of purified Pms mycelial walls is presented in Table I. The elements C, H, O, and N comprise 57.3% of the walls and the remainder is accounted for by water (4.4%) and ash (35.4%).

The carbohydrate content of purified Pms mycelial walls was measured by a modification of the anthrone assay (7). Ten mg of air-dried mycelial walls were dissolved in 10 ml of 67% (v/v) sulfuric acid. This solution was diluted to 10% of its concentration with H_2O and the carbohydrate content was determined by the anthrone procedure (7). The carbohydrate content of purified mycelial walls of Pms, based on several determinations, was 60%.

The protein content of mycelial walls was determined by a modification of the method of Lowry *et al.* (14). Ten mg of purified mycelial walls were suspended for 1 hr at 60 C in 10 ml of 2% (w/v) Na_2CO_3 in 0.1 N NaOH. This treatment solubilized the protein present in the mycelial walls. The insoluble material was removed by centrifugation and the protein content of the supernatant liquid was determined by the method of Lowry *et al.* (14). The protein content of purified mycelial walls based on this method was 6.7%. The protein content was also determined indirectly by multiplying the nitrogen content by 6.25 giving a value of 6.6%. Discounting the H_2O and the inorganic salts, the mycelial walls consist of 90% polysaccharide and 10% protein.

Table I. *Chemical Composition of Pms Mycelial Walls*

Component	% Composition
Carbon	17.9
Hydrogen	4.5
Oxygen	33.8
Nitrogen	1.06
Ash	35.4
Total	92.66
Total carbohydrate (anthrone)	60
Total protein (Lowry)	6.7
Total protein (N × 6.25)	6.6
Ash	35.4
H_2O	4.4

The sugar composition of purified mycelial walls of Pms was determined by the alditol acetate method (9). The Pms wall polysaccharides are 95% glucose, 3% mannose, and 2% other sugars including hexosamine (<1%).

Extraction of Elicitor from Purified Mycelial Walls of Pms. Elicitor was extracted from purified Pms mycelial walls by a technique used to release the surface antigens of yeast (16). Mycelial walls were suspended in H_2O (100 ml/g of walls), and the suspension was heated under pressure at 121 C for 3 hr. The solubilized elicitor was separated from the wall residue by centrifugation followed by passage through a membrane filter (0.22 μm, Millipore). The heat-solubilized wall fraction was concentrated to 1% of its original volume by rotary evaporation at 40 C under reduced pressure and dialyzed against three changes of 8 liters of deionized H_2O. The heat-solubilized, nondialyzable material obtained from purified walls of each of the three races of Pms were the sources of wall-released elicitor used in this investigation.

The yield of wall-released elicitor from 6.6 g of purified Pms mycelial walls (discounting salts and H_2O) is routinely 290 mg of carbohydrate and 300 mg of protein. The wall-released elicitor, therefore, represents 4.8% of the wall polysaccharide and 43% of the wall protein or 8.9% of the organic matter of the mycelial wall. Wall-released elicitor carbohydrate is composed of 34% glucose, 50% mannose, and 10% glucosamine. The mannose present in this fraction represents 90% of that present in the walls. Similarly, glucosamine, which makes up less than 1% of the walls, makes up 10% of the composition of wall-released elicitor, indicating that at least 48% of the glucosamine content of the mycelial wall is released by heat treatment.

Fractionation of Wall-released Elicitor. The wall-released elicitor preparations from the three races of Pms were each fractionated on the basis of their affinity for the anion exchanger DEAE-cellulose (Whatman). A sample of wall-released elicitor containing 100 mg of carbohydrate and 100 mg of protein in 100 ml of 10 mM potassium phosphate, pH 8, was applied to a column (1.8 × 13 cm) of DEAE-cellulose which had been equilibrated with 10 mM potassium phosphate, pH 8. The column was washed with 100 ml of the equilibration buffer, and the bound material was eluted from the column by a linear 0 to 1 M NaCl gradient prepared in the same buffer (total gradient volume was 500 ml) (Fig. 1). Elicitor activity was associated both with that fraction which had no affinity for the column and with the material which was bound to the column and was subsequently eluted from the column in the NaCl gradient.

Chromatography on the DEAE-cellulose column divided the wall-released elicitor into two fractions based on the anionic nature of the elicitor. Since there was no separation of active and inactive material using the NaCl gradient, the gradient was replaced in all subsequent fractionations by a single wash with 200 ml of 1 M NaCl in 10 mM potassium phosphate, pH 8. The void or unbound fraction was contained in the first 28 fractions of the column effluent. The bound fraction consisted of the effluent from the NaCl gradient contained in fractions 29 through 62. The separate void and bound fractions from each Pms race were dialyzed against two changes of 8 liters of deionized H_2O. The void fraction contained 49% of the carbohydrate and 35% of the protein of the wall-released elicitor preparation. The bound fraction contained the remainder of this material.

Two fractions (void and bound) were obtained from the DEAE-cellulose chromatography of the wall-released elicitor from each of the three races of Pms. These fractions were further fractionated by affinity chromatography using a column of concanavalin A covalently linked to Sepharose (Pharmacia). The DEAE-cellulose void and bound fractions of the wall-released elicitor were each evaporated to dryness under reduced pressure at 40 C. The dried residues were dissolved in 100 ml of Con A buffer (100 mM potassium phosphate, pH 7.2, containing 10 μM

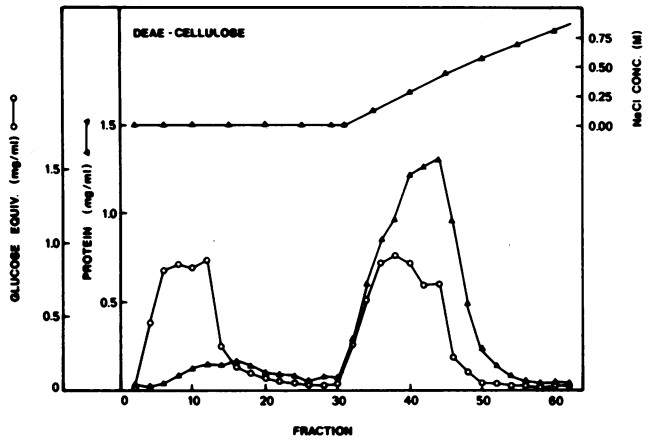

FIG. 1. DEAE-cellulose chromatography of elicitor released from purified mycelial walls of Pms. The wall-released elicitor from Pms race 1 (100 mg of carbohydrate, 100 mg of protein) was applied to a column (2.6 × 16 cm) of DEAE-cellulose in 10 mM sodium phosphate, pH 8, and the column was washed with this buffer. A linear 0 to 1 M NaCl gradient prepared in the same buffer was applied in a total of 500 ml. Fractions were 10 ml. The NaCl concentration (▲) of the effluent was determined by conductivity. The carbohydrate (○) and the protein (△) contents were determined as described under "Materials and Methods."

$MnCl_2$, 10 μM $CaCl_2$, and 0.01% (w/v) Thimerosal [Sigma]) and passed individually through a column (1.5 × 13 cm) of concanavalin A-Sepharose which had been previously washed with at least 1 liter of Con A buffer. After the samples were applied, the columns were washed with 100 ml of Con A buffer followed by 100 ml of a 4% (w/v) solution of α-methyl-D-mannopyranoside (Sigma) in the Con A buffer. The effluent from the sample application combined with the buffer wash was designated the concanavalin A-void fraction. The effluent from the α-D-mannopyranoside wash was designated the concanavalin A-bound fraction. An example of the fractionation using concanavalin A-Sepharose is presented in Figure 2.

A small amount of concanavalin A (less than 0.1 μg/ml of column effluent) was released from the concanavalin A-Sepharose column using the conditions described. To avoid any complications in the elicitor assays due to the binding activity of the eluted concanavalin A, all samples were heated at 121 C for 30 min to inactivate any contaminating concanavalin A.

The combination of chromatography on DEAE-cellulose and concanavalin A-Sepharose resulted in four fractions from each preparation of wall-released elicitor. The material without affinity for DEAE-cellulose or for concanavalin A-Sepharose is designated Fraction I. Fraction II is bound by concanavalin A Sepharose but not by DEAE-cellulose. Conversely, Fraction III is bound to DEAE-cellulose, but not by concanavalin A-Sepharose. The portion of the wall-released elicitor which binds to both columns is designated as Fraction IV.

The distribution of the protein and carbohydrate from the mycelial wall-released elicitor into each of the four fractions is presented in Table II. The distribution of material into the four fractions is similar for all three races of Pms. The percentage of the total carbohydrate and protein of the mycelial walls which is found in each fraction (Table II) is also similar.

Abilities of Fractions I, II, III, and IV to Elicit Glyceollin Accumulation in Soybean Tissues. Fractions I, II, III, and IV from Pms races 1, 2, and 3 were tested for their abilities to stimulate glyceollin accumulation in cotyledons and hypocotyls of Harosoy 63 soybean seedlings. The elicitor activity of each of the fractions was determined for several different amounts of elicitor applied to the two soybean tissues. The elicitor amounts are expressed in terms of μg of carbohydrate, as this value appears to be more related to the activity of the elicitor fractions than total weight of the fractions. In other words, the protein content of the fractions appears to be unrelated to elicitor activity.

The elicitor activity (A_{285}) in the cotyledon assay of various amounts of the corresponding fractions from each of the three races of Pms is presented in Figure 3. The application to different sets of cotyledons of equal amounts of the corresponding fractions from each race, *e.g.* fraction I of races 1, 2, and 3, resulted in the accumulation of equal amounts of glyceollin. Different fractions from the same race, *e.g.* fractions I, II, III, and IV of race I, did not result in the accumulation of equivalent amounts of glyceollin. Fractions I, II, III, and IV of any one race are not, on a weight basis, equally effective elicitors. One way of comparing the relative activities of the fractions is by determining the amount (μg of carbohydrate) of each fraction required to give an arbitrary A_{285} value. The values obtained by this type of calculation are inversely proportional to the relative elicitor activity of each fraction. The amount of each fraction required to

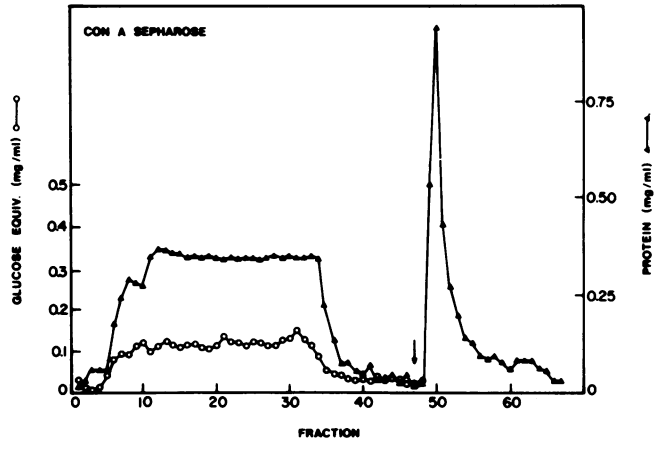

FIG. 2. Concanavalin A-Sepharose affinity chromatography of Pms wall-released elicitor. The DEAE-cellulose bound fraction of the wall-released elicitor from Pms (race 1) was applied in 100 ml of Con A buffer (see text) to 20 ml of concanavalin A-Sepharose and 5.2-ml fractions were collected. The column was washed with 100 ml of the Con A buffer. The void volume was contained in fractions 1–4. The bound material was removed with 100 ml of the buffer containing 4% (w/v) α-methyl-D-mannopyranoside (arrow). Column fractions 48–65 represent the concanavalin A-bound fractions. Fractions 49+ contained α-methyl-D-mannopyranoside which prevented direct measurements of the carbohydrate content of these fractions. The carbohydrate (○) and protein (△) content of each fraction were determined as described under "Materials and Methods."

Table II. *Distribution of Protein and Carbohydrate from Mycelial Wall-released Elicitor into Each of Four Fractions*

WALL-RELEASED ELICITOR FRACTION	RACE	% OF TOTAL WALL-RELEASED ELICITOR PRESENT IN EACH FRACTION		% OF TOTAL WALL ACCOUNTED FOR BY EACH FRACTION	
		CARBOHYDRATE	PROTEIN	CARBOHYDRATE	PROTEIN
I	1	40.1	9.3	2.1	4.0
	2	39.7	10.6	2.2	4.6
	3	56.9	17.0	2.2	7.3
II	1	2.5	2.2	0.2	0.9
	2	2.9	3.0	0.2	1.3
	3	4.8	6.7	0.2	2.9
III	1	43.1	68.0	1.6	29.2
	2	42.3	62.9	1.8	27.0
	3	23.9	51.8	1.8	22.3
IV	1	14.4	20.5	0.7	8.8
	2	15.1	23.9	0.5	10.1
	3	14.4	24.5	0.6	10.5

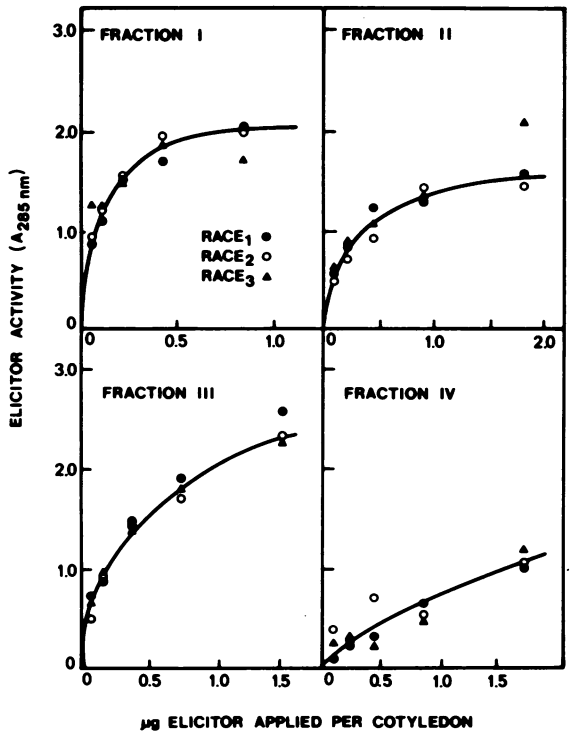

FIG. 3. Elicitor activity of wall-released fractions I, II, III, and IV from Pms races 1, 2, and 3 as determined by the cotyledon assay. A series of increasing amounts of each elicitor fraction was applied to different sets of cotyledons and the activity (A_{285}) was measured. The μg of elicitor listed represent μg of elicitor carbohydrate (see text). Each datum is the average of three determinations. Corresponding fractions of wall-released elicitor were obtained from Pms races 1 (●), 2 (○), and 3 (▲).

give an A_{285} value of 1 is: I, 0.06; II, 0.35; III, 0.20; and IV, 1.46 μg carbohydrate/cotyledon (Fig. 3). The relative activities of the four fractions, normalized to the activity of fraction I are: I, 100; II, 17; III, 29; and IV, 4.

The four wall-released fractions from each race of Pms were also assayed for their abilities to stimulate glyceollin accumulation in soybean hypocotyls. Each fraction of the three races was applied in several concentrations to sets of wounded hypocotyls and the glyceollin content of the treated hypocotyls was measured (Fig. 4). The application to the hypocotyls of equal amounts of the corresponding fractions from each race of Pms, *e.g.* fraction I from races 1, 2, and 3, resulted in the accumulation of equal amounts of glyceollin (Fig. 4). The four fractions from the same race, *e.g.* fractions I, II, III, and IV from race 1, did not result in the accumulation in the hypocotyls of equal amounts of glyceollin. As in the cotyledon assay, the four wall-released elicitor fractions have different relative activities in the hypocotyl assay. The amounts (μg carbohydrate/hypocotyl) of each wall-released fraction required to elicit the production of 75 nmoles of glyceollin/hypocotyl are: I, 0.3; II, 2.1; III, 1.7; and IV, 4.3. The relative activities of the fractions normalized to fraction I are: I, 100; II, 14; III, 21; and IV, 7.

The response of soybean cell suspension cultures to various concentrations of elicitor fractions I and IV was measured by the stimulated increase in phenylalanine ammonia-lyase activity in elicitor-treated cultures (8). There is an optimum concentration of each fraction for maximal stimulation of phenylalanine ammonia-lyase activity in the cell suspension cultures. The optimum concentrations were 1 and 10 μg of carbohydrate/ml for fractions I and IV, respectively. Since the optimum concentrations are inversely proportional to the relative activities of these fractions, the relative activities of fractions I and IV, normalized to fraction I, are 100 and 10, respectively.

Comparison of Abilities of Live Pms and Fraction I Elicitor to Stimulate Glyceollin Accumulation in Soybean Hypocotyls. Soybean hypocotyls accumulate glyceollin when inoculated with living mycelia of Pms or when treated with elicitor isolated from the mycelial walls of Pms. If the wall-released elicitor is present in the living mycelia and is detected by the plant when the plant is inoculated with Pms mycelia, then the accumulation of glyceollin in seedlings inoculated with Pms mycelia would be similar to that in seedlings treated with elicitor. This hypothesis was tested experimentally.

Pms mycelia from 5-day-old cultures grown on V8 broth at 26 C were prepared for inoculation. The mycelia were washed by filtration on a coarse sintered-glass funnel with 50 ml of 10 mM sodium phosphate, pH 7.2. The washed mycelia were then homogenized in 10 ml of the buffer for 5 sec at maximum speed in a Sorvall Omni-Mixer. The mycelial fragments were washed three times with 25 ml of the 10 mM buffer, collecting the mycelia each time by suction filtration on the sintered-glass funnel. The washed mycelia were resuspended in 5 ml of the 10 mM phosphate buffer containing 200 μg/ml penicillin G (Sigma). Twenty-five μl of this suspension of live mycelial fragments were applied to each hypocotyl, which had been wounded as in the standard hypocotyl assay (3). Harosoy 63 seedlings inoculated in this manner with mycelia from an incompatible race of Pms (race 1) exhibited, after 48 hr of incubation at 26 C in the dark, the typical minor symptoms associated with an incompatible interaction. Harosoy 63 seedlings inoculated with a compatible race of

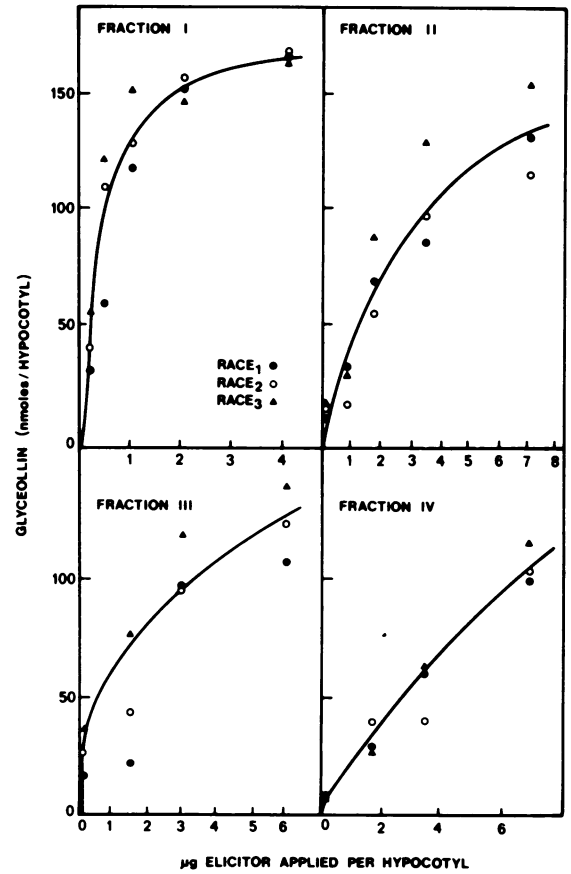

FIG. 4. Elicitor activity of wall-released elicitor fractions I, II, III, and IV from Pms races 1, 2, and 3 as determined by the hypocotyl assay. Each elicitor fraction of the three Pms races was applied in several concentrations to sets of hypocotyls and the activity (nmoles of glyceollin per hypocotyl) was measured. The amounts of elicitor listed represent μg of elicitor carbohydrate (see text). Corresponding fractions of wall-released elicitor were obtained from Pms races 1 (●), 2 (○), and 3 (▲).

Pms (race 3) exhibited advanced rot symptoms, water soaking, and collapse of the hypocotyls accompanied by wilting of the leaves. The inoculation technique retains the characteristics of a natural infection with a compatible or an incompatible race of Pms.

This hypocotyl assay was used to compare the relative effectiveness of live mycelia and elicitor in stimulating glyceollin accumulation. Each of a set of Harosoy 63 seedlings was inoculated with a mycelial suspension of an incompatible race of Pms (race 1). Fraction I elicitor (200 μg carbohydrate/ml in 10 mM sodium phosphate, pH 7.2, containing 100 μg/ml penicillin G) was applied in 25 μl to each of an equivalent set of wounded Harosoy 63 seedling hypocotyls. The Pms-inoculated and the elicitor-treated hypocotyls were then assayed for glyceollin content (3) after various periods of incubation (Fig. 5). The rate of glyceollin accumulation in seedlings treated with incompatible mycelia or with elicitor was indistinguishable. The rate of glyceollin accumulation was also determined for soybean seedlings inoculated with a suspension of compatible Pms mycelia (from race 3). The onset and the rate of glyceollin accumulation in seedlings inoculated with compatible mycelia was indistinguishable from the onset and the rate of glyceollin accumulation in seedlings inoculated with either incompatible mycelia or purified elicitor. This experiment was repeated giving essentially the identical result.

Effect of Adding Purified Elicitor to Soybean Seedlings Inoculated with Live Pms. The presence or absence of elicitor during an infection may determine whether glyceollin is synthesized and consequently whether a pathogen yields an incompatible or compatible reaction with the host. Elicitor fraction I (4 μg carbohydrate/hypocotyl) and a suspension of mycelia from a compatible race of Pms (race 3) were applied simultaneously to Harosoy 63 hypocotyls and the hypocotyls were incubated for 48 hr to determine if the presence of elicitor was sufficient to make a normally compatible host-pathogen interaction incompatible. The seedlings which received elicitor and mycelia simultaneously were compared to other seedlings which were treated with water or which received only elicitor or only the compatible mycelia. The wounds of seedlings receiving only elicitor became darkly pigmented, but otherwise the seedlings were as healthy in appearance as those treated with water. The seedlings which received elicitor and mycelia simultaneously were identical with those which received only mycelia; both sets of seedlings were completely flaccid with advanced symptoms of rot. Elicitor applied simultaneously with mycelia could not, therefore, protect the plants from the compatible pathogen.

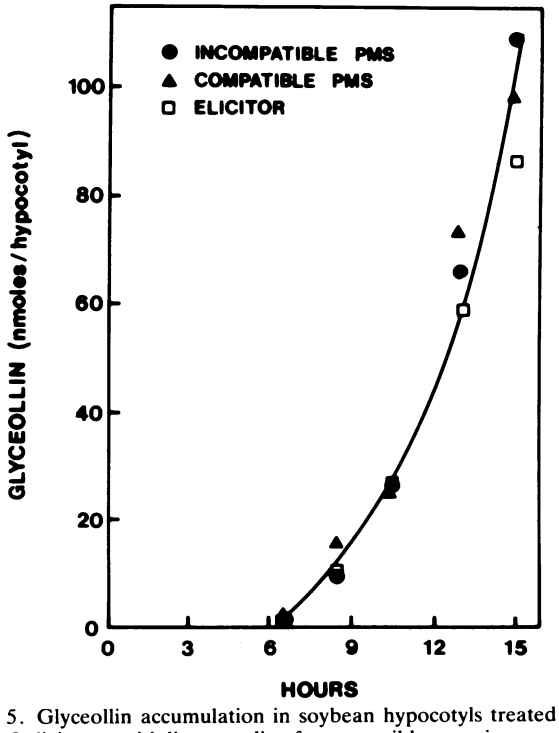

FIG. 5. Glyceollin accumulation in soybean hypocotyls treated with fraction I elicitor or with live mycelia of a compatible or an incompatible race of Pms. Wounded hypocotyls (Harosoy 63) were treated with fraction I elicitor (4 μg of carbohydrate/hypocotyl, □) or were inoculated with live mycelial fragments of compatible (race 3, ▲) or incompatible (race 1, ●) races of Pms. The glyceollin content of the hypocotyls was determined (3) after various intervals of incubation at 100% humidity in the dark at 26 C.

This inability of elicitor to protect the plant could be explained by the inability of glyceollin to accumulate rapidly enough to inhibit the spread of a compatible race of Pms. It was previously observed (3) that glyceollin accumulation reached a level toxic to Pms in 10 to 12 hr after the application of elicitor to soybean tissues. Mycelia of Pms applied to soybean hypocotyls which had been treated with elicitor 10 hr previously should, therefore, be unable to colonize the protected tissue. Elicitor fraction I (4 μg carbohydrate/hypocotyl) was applied to wounded hypocotyls of soybean seedlings and 10 hr later 25 μl of a mycelial suspension from a compatible race of Pms (race 3) was applied to the same sites. These plants were compared 48 hr later to control sets of soybean seedlings which received water in place of elicitor. The plants pretreated with water followed 10 hr later by mycelia from the compatible race of Pms exhibited advanced rot symptoms, but the plants which received a pretreatment with elicitor prior to the mycelia exhibited only the minor symptoms associated with an incompatible reaction. Elicitor is, therefore, capable of protecting soybean hypocotyls from infection by a compatible race of Pms if the elicitor is applied to the hypocotyls 10 hr prior to inoculation with the pathogen.

DISCUSSION

The purified mycelial walls of Pms are 60% carbohydrate, 6.7% protein, and the remainder is water and insoluble salts not removed during processing. Discounting the water and salts, the walls are 90% carbohydrate and 10% protein. The carbohydrate and protein composition of these purified mycelial walls of Pms is similar to that determined for the closely related fungus *Phytophthora cinnamomi* (6). The sugar composition of the mycelial walls of Pms is 95% glucose and 3% mannose, with other sugars present in only minor amounts. This composition is also similar to that determined for *P. cinnamomi* (6). The material in the total wall-released elicitor fraction described in the present paper accounts for only 8.9% of the organic matter of purified mycelial walls, yet this fraction contains 90% of the mannose, at least 48% of the glucosamine and 43% of the protein present in the mycelial walls. Thus, the wall-released elicitor fraction represents a discrete portion of the mycelial wall. However, it has not been determined whether additional elicitor remains in the residue of the wall.

The extraction conditions (121 C for 3 hr) used to solubilize elicitor from the purified mycelial walls of Pms were originally used by Raschke and Ballou (16) to isolate the surface antigens from yeast. Yeast cell walls contain a rigid layer of β-1,3-linked glucan, a mannan-protein complex and mannan-protein enzymes (5). The heat treatment solubilizes the mannan-protein complex and the mannan-protein enzymes of yeast cell walls. The mannan portion of this yeast fraction is the immunodominant component of the cell surface (5). The heat treatment of purified Pms mycelial walls solubilizes a fraction, the wall-released elicitor, which is rich in mannan and protein. This similarity is enhanced by the isolation of a fraction of the wall-released elicitor, fraction IV, which accounts for only 0.8% of the wall

organic matter but which contains 20% of the total mannose of the wall (4). Like the yeast, the mannose of fraction IV is present as a mannan-containing glycoprotein (4). Generalizing from the characteristics of yeast cell walls, it is likely that the wall-released elicitor contains a surface component of Pms mycelia.

The mycelial wall-released elicitor was fractionated on the basis of affinity for an anion exchange column (DEAE-cellulose) and for concanavalin A-Sepharose. Four fractions were obtained. Fraction I has properties very similar to the extracellular elicitor from cultures of Pms. It has no affinity for either DEAE-cellulose or concanavalin A. The other three fractions exhibit affinity for DEAE-cellulose (III) or concanavalin A-Sepharose (II) or both (IV). The fact that all four fractions exhibit elicitor activity suggests that the same moiety is responsible for elicitor activity in each of these fractions and that this moiety is covalently attached to other molecules with different chemical characteristics. These elicitor-containing molecules have presumably been fractionated on the basis of the portions of the molecules which do not have elicitor activity. The active moiety common to all four fractions is likely to be the glucan, which is present in all four fractions and is the predominant component of fraction I and of the purified extracellular elicitor (3, 4). The less likely alternative to this explanation of the varying character of the elicitor molecules is that plants are able to recognize several different elicitors in the wall-released elicitor preparations.

The four fractions from each of the three races of Pms were assayed for elicitor activity on cotyledons and hypocotyls. The equivalent fractions from each of the races give equivalent responses in each of the assays. The relative activities of the wall-released elicitor fractions are also very similar in each assay: if fraction I is given a relative activity of 100 in the cotyledon, hypocotyl, and cell suspension culture assays, then fraction II is 17% as active in the cotyledon assay and 14% as active in the hypocotyl assay; fraction III is 21% as active in the cotyledon assay and 29% as active in the hypocotyl assay; and fraction IV is 4% as active in the cotyledon assay, 7% as active in the hypocotyl assay and 10% as active in the cell-suspension culture assay. These findings confirm that the elicitors from the three races are identical in biological activity, that the fractionation procedures used give reproducible results, and that the three assays are equally valid measures of elicitor activity.

The rate of glyceollin accumulation is essentially the same in soybean hypocotyls treated with elicitor or inoculated with mycelia from compatible or incompatible races of Pms (Fig. 5). This observation suggests that elicitor plays a role in the interactions of soybeans with both compatible and incompatible races of Pms. If this is true, the presence or absence of elicitor could not determine whether a particular soybean-Pms combination would result in a compatible or an incompatible interaction (11).

Another demonstration that the presence of the elicitor is not sufficient to determine race-specific resistance was derived from experiments in which elicitor was applied simultaneously with mycelia of a compatible race of Pms to wounded hypocotyls. The presence of elicitor did not stop the growth of the compatible race of Pms. Pretreatment of hypocotyls with elicitor 10 hr prior to inoculation with a compatible race of Pms protected the seedlings against the spread of the pathogen. These findings indicate that soybean seedlings require time to respond to elicitor before the compatible race of Pms becomes established. This is consistent with the hypothesis that elicitors are active during infections involving both compatible and incompatible races of Pms and that the distinction between compatible and incompatible interactions is in the rate at which the pathogen is able to colonize new tissues of its host. The incompatible races of Pms may succumb to glyceollin because they do not colonize new tissue with sufficient speed. Compatible races of Pms, on the other hand, may avoid the toxic effects of glyceollin by colonizing new tissues before toxic glyceollin levels are reached.

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