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Host-Pathogen Interactions

IX. QUANTITATIVE ASSAYS OF ELICITOR ACTIVITY AND CHARACTERIZATION OF THE ELICITOR PRESENT IN THE EXTRACELLULAR MEDIUM OF CULTURES OF *PHYTOPHTHORA MEGASPERMA* VAR. *SOJAE*[1]

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ABSTRACT

Resistance of soybean (*Glycine max* L.) seedlings to *Phytophthora megasperma* var. *sojae* (Pms) is in part due to the accumulation in infected tissue of a compound which is toxic to Pms. The accumulation of this compound, a phytoalexin called glyceollin, is triggered by infection, but it can also be triggered by molecules, "elicitors," present in cultures of Pms. The ability of the Pms elicitor to stimulate phytoalexin accumulation in soybean tissues has been used as the basis for biological assays of elicitor activity. Two bioassays were developed and characterized in this study of the Pms elicitor. These bioassays use the cotyledons and the hypocotyls of soybean seedlings. The cotyledon assay was used to characterize the extracellular Pms elicitor. This elicitor was isolated from Pms cultures and purified by ion exchange and molecular sieving chromatography. The extracellular Pms elicitor was determined to be a predominantly 3-linked glucan, which is similar in composition and structure to a polysaccharide component of Pms mycelial walls.

One of the most common reactions of a plant in response to invasion by a pathogen involves the precipitous death or hypersensitive response of those plant cells which have come in contact with a pathogen (9, 25, 32, 33). The direct effects of plant cell death on pathogen development are not known. It has been suggested that cell death may result in the disruption of lysozymes and other cellular structures, releasing enzymes and compounds which may interfere with the growth of the pathogen (37). Compounds which are toxic to the pathogen are present in the area of localized necrosis, but these compounds, called phytoalexins, are thought to be synthesized by the healthy cells neighboring the necrotic area (25, 29).

It is not known how hypersensitive cell death and phytoalexin accumulation are initiated. The first clues to the nature of the molecules which trigger the accumulation of phytoalexins came from studies of pathogens grown in defined media. Cell-free filtrates from cultures of several pathogens (1-3, 12, 13, 19, 22, 28, 30, 35, 36) were demonstrated to elicit phytoalexin accumulation in their hosts. One of the first of these reports was in 1958 by Uehara (35) who applied filtrates from cultures of *Fusarium* sp. to seed pods of soybeans, the host of this fungal pathogen, and observed that a fungitoxic compound accumulated in the bean tissue. Similar observations of phytoalexin accumulation in green bean tissue have been made after application of culture filtrates of *Monilinia fructicola* (7), *Penicillium expansum* (30), and *Colletotrichum lindemuthianum* (1, 2). The cell-free culture filtrates of the fungal pathogen (*Phytophthora megasperma* var. *sojae* have also been shown to stimulate phytoalexin accumulation in its host, soybeans (3, 12, 13, 17, 19, 22, 28). Attempts were made to purify from some of these pathogen cultures the molecules responsible for triggering phytoalexin accumulation; with the exception of the glucan produced by *C. lindemuthianum* (2), these efforts were unsuccessful. The pathogen-synthesized molecules responsible for stimulating accumulation of phytoalexins in their hosts are termed "elicitors" (19).

The only other elicitor that has been purified was obtained from the mycelia of *M. fructicola* (8), a fungal pathogen of stone fruit trees. The *M. fructicola* mycelial elicitor, called monilicolin A, is reported to be a small peptide and is active on a tissue of the green bean, a nonhost of *M. fructicola* (8). It is doubtful that monilicolin A has a physiological role in disease resistance for this elicitor has been demonstrated only to be active on a single nonhost of *M. fructicola*. Other nonhost species, which are resistant to *M. fructicola*, do not accumulate phytoalexin in response to this peptide (8), and no host of this pathogen has been shown to be responsive to the elicitor.

Phytoalexin accumulation in response to monilicolin A may actually reflect a relatively nonspecific disruption of normal plant metabolism. There are abundant examples of the triggering of precise biochemical events by nonspecific mechanical or chemical means. The induction of lytic reproduction of prophage in response to UV radiation (24) and the mechanical induction of parthenogenesis in amphibian eggs (34) are two of the many examples. If monilicolin A is nonspecific, it would be only one of a broad spectrum of nonspecific reagents capable of inducing phytoalexin accumulation in plants. Other such reagents include the salts of heavy metals (14), inhibitors of RNA and protein synthesis (31), and compounds which intercalate in DNA (15). These chemicals are not normally encountered by plant cells, especially not in the relatively high concentrations required.

It is important to look for elicitors which are likely to be accessible to the host plant and which are effective in physiological concentrations. One possible source of such elicitors is the cell-free media of cultured pathogens, for these extracellular elicitors are likely to be released by pathogens when growing in a plant. This possibility and the desire to know the chemical nature of an elicitor prompted the purification and characterization reported in this paper of the elicitor present in the culture filtrates of Pms,[5] the fungal pathogen which causes root and stem rot in soybeans.

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

Several semiquantitative bioassays for elicitor activity have been described (13, 19, 21), but none of these assays was sufficiently quantitative to permit its use in the purification of an elicitor. The assays described here are major refinements and modifications of two assays partially characterized by other workers. These assays use cotyledons and hypocotyls from soybean seedlings. The accumulation of the soybean phytoalexin glyceollin (6) in soybean cotyledons and hypocotyls treated with elicitor preparations is used as a measure of elicitor activity. In addition, a completely new elicitor assay utilizing soybean cell suspension cultures has been developed and will be described in a subsequent paper (11). Together, these three assays permitted the advances in our understanding of the molecular biology of host-parasite interactions which are to be presented.

MATERIALS AND METHODS

Chemical Assays. Neutral sugars were assayed by the anthrone test as described by Dische (10). Protein was assayed as described by Lowry *et al.* (23). Purification of the elicitor used for this paper (mycelial wall-released elicitor fraction I from Pms race 1) is described in the following paper (4). Elicitor amounts are expressed as the μg equivalents of glucose required to give the same colorimetric reading (A_{620}) in the anthrone assay as the elicitor preparations.

The techniques used to obtain the sugar and the glycosyl linkage composition of polysaccharides are presented in a subsequent paper (5).

Soybean Seedlings. Seeds of soybean (*Glycine max* L. cv. Harosoy 63) were obtained from the U. S. Regional Soybean Research Laboratory, Urbana, Ill., from Wilken Seed Grains, Pontiac, Ill., and from Dr. J. Paxton, Department of Plant Pathology, University of Illinois, Urbana. The seeds were surface-sterilized by soaking in 0.75% sodium hypochlorite (15% Clorox) for 5 min and were extensively rinsed with distilled H_2O. The sterilized seeds were planted between two 1-cm layers of vermiculite on a 9-cm layer of rich potting soil. Seedlings were grown in a Percival growth chamber and were watered daily with tap H_2O. A photoperiod regime of 14 hr at 2500 ft-c of illumination and 10 hr of darkness was employed. The temperatures during the light and dark cycles were 28 and 22 C, respectively. The relative humidity was maintained at approximately 75%.

Pathogen Culture. *Phytophthora megasperma* Drechs, var. *sojae* A. A. Hildb. races 1, 2, and 3 were obtained from N. T. Keen, University of California, Riverside. Cultures were maintained on V8 agar slants or plates. Large cultures were grown in 2.8-liter Fernbach flasks containing 500 ml of asparagine medium (17). The liquid cultures were inoculated with 4-mm diameter mycelial agar plugs cut from the periphery of mycelia growing radially on agar plates. On the 2nd and 4th day after inoculation, the liquid cultures were vigorously swirled by hand for approximately 15 sec to aid the spread of the mycelia on the surface of the media. Cultures were incubated at 24 C without further agitation until the mycelia covered the surface of the liquid (14–21 days).

Purification, Characterization, and Quantitation of Glyceollin. Glyceollin was produced in bulk by a modification of the method described by Keen (18). Soybean seeds (500 g) were soaked in distilled H_2O for 4 hr at 21 C and sliced into quarters. The sliced seeds were then sprayed with conidia (approximately 10^7 in 20 ml of H_2O) of *Cladosporium cucumerinum*, a nonpathogen of soybeans, and the inoculated seeds were stored in the dark at 21 C for 72 hr. The cut surfaces turned a deep red during the incubation, a response that has been correlated with but is not due to the accumulation of glyceollin. Glyceollin is a colorless compound.

The infected seeds were soaked in 500 ml of 95% ethanol for 48 hr at 4 C to extract glyceollin. The ethanol extract was dried under reduced pressure by rotary evaporation at 40 C, and the residue was dissolved in 10 ml of ethanol. The glyceollin solution was applied in 0.5-ml aliquots to preparative TLC plates of silica gel (PF_{254}, Van Waters and Rogers). The plates were developed with solvent 1, hexane-ethyl acetate-methanol (60:40:5, v/v). Glyceollin was identified as a UV-absorbing compound, R_F value of 0.32, and by its fungitoxicity in the TLC-*C. cucumerinum* assay (20). The UV-absorbing band containing glyceollin was scraped from each of 20 preparative plates, washed from the silica with 95% ethanol, and dried by rotary evaporation under reduced pressure. The glyceollin was further purified by preparative TLC using solvent 2, benzene-methanol (95:5, v/v). The glyceollin, which has an R_f of 0.21 in this solvent, was recovered from the silica in 95% ethanol, dried under reduced pressure, and redissolved in 5 ml of methanol.

The highly purified glyceollin was still further purified by chromatography on Sephadex LH-20 (Pharmacia). The methanol solution of glyceollin was passed in 1-ml lots through a column (2 × 30 cm) of Sephadex LH-20, and the column was eluted with methanol. The glyceollin was detected in the column effluent by its absorbance at 285 nm. The column fractions with high absorbance at 285 nm were combined and dried by rotary evaporation. Residual methanol was removed by addition of 25 ml of diethyl ether followed by rotary evaporation to dryness.

The purity of the glyceollin obtained by this procedure was confirmed by TLC using two solvents: toluene-chloroform-acetone (40:25:35, v/v) and benzene-dioxane-acetic acid (90:25:4, v/v) in addition to solvents 1 and 2. The yield from 500 g of soybean seeds was 300 mg of glyceollin. The molar extinction coefficient at 285 nm in ethanol was ϵ = 10,300. Mass spectrometry yielded m/e peaks at 338, 323, 309, 305, 295, 277, 173, and 153. These data are consistent with the findings of Burden and Bailey (6) for glyceollin.

Carbohydrates. Yeast mannan, larch galactan, laminarin, nigeran, and α-methyl-D-galactopyranoside were purchased from Koch-Light Laboratories. Lactose, maltose, cellobiose, α-methyl-D-mannopyranoside, and mannose were purchased from Sigma Chemical Co. α-Methyl-D-glucopyranoside and β-methyl-D-glucopyranoside were purchased from Nutritional Biochemical Corp. Galactose and glucose were purchased from Fisher Scientific Co.

RESULTS

Cotyledon Assay of Elicitor Activity. Soybean tissues accumulate glyceollin in response to elicitors. This response is the basis of several biological assays. The assay described here was developed from the cotyledon assay first used by Frank and Paxton (13).

Cotyledons, detached from 8-day-old seedlings of the soybean cultivar Harosoy 63, were soaked for 5 min in 0.75% sodium hypochlorite (15% Clorox) and were extensively washed with distilled H_2O. A section, approximately 1 mm thick and 6 mm in diameter, was cut from the upper surface of each cotyledon. A margin of intact cuticle, approximately 2 mm wide, remained around the resulting wound. The sample to be assayed for its ability to elicit the accumulation of the soybean phytoalexin, glyceollin, was diluted with 10 mM sodium phosphate, pH 7.2, containing 100 μg/ml of the antibiotic gentamicin sulfate (Schering). A 100-μl drop of the sample solution was applied to each of a set of 10 wounded cotyledons. The treated cotyledons were incubated on moist filter paper discs in covered Petri plates at 26 C. After 20 hr, each set of 10 cotyledons was transferred with forceps to 20 ml of distilled H_2O contained in a 50-ml Erlenmeyer flask. This procedure rinses off the droplets of sample fluid that are retained on the wound surfaces. All measurements are presented in terms of this wound-droplet solution. It will be shown below that the A_{285} of the wound-droplet solution is a

measure of elicitor activity. The data presented are the averages of three separate determinations.

Correlation between A_{285} Measurements and Glyceollin Content in Cotyledon Assay. This assay uses the absorbance of wound-droplet solutions at 285 nm (A_{285}) as a measure of glyceollin content and thereby of elicitor activity. For this to be a valid assay, it was necessary to demonstrate that a correlation exists between A_{285} measurements and the glyceollin content of these solutions.

Glyceollin absorbs light at 285 nm. The contribution of glyceollin to the A_{285} of wound-droplet solutions was determined by the following experiment. An amount of elicitor, in the range of 0.05 to 0.8 μg, was applied to each of a set of 10 wounded cotyledons and, after incubation, the wound-droplet solutions were assayed both for absorbance at 285 nm and for their glyceollin content (Fig. 1). The glyceollin content of the wound-droplet solution was determined by the following method. Five ml of wound-droplet solution and 5 ml of ethyl acetate were combined in a test tube and thoroughly mixed. Three ml of the ethyl acetate layer were transferred to another test tube and evaporated to dryness under a stream of filtered air. The residue was dissolved in 200 μl of ethyl acetate and a 100-μl aliquot (50% of the sample) was applied to a preparative TLC plate of silica gel. The UV-absorbing spot, with the same R_F as an authentic glyceollin standard, was scraped from the plate, dissolved in 5 ml of 95% ethanol, and A_{285} was measured. Using the molar extinction coefficient (ϵ = 10,300 in ethanol), the glyceollin content of the wound-droplet solution was calculated. Figure 1 demonstrates that the A_{285} and the glyceollin content of wound-droplet solutions have a linear relationship. The glyceollin present in the wound-droplet solution is responsible for 24% of the A_{285} using the range of elicitor amounts and the assay conditions described above.

Rate of Glyceollin Accumulation of Elicitor-treated Cotyledons. The rate at which glyceollin accumulates in soybean cotyledons following treatment of the cotyledons with Pms elicitor was investigated in order to determine a suitable incubation time for elicitor assays. The glyceollin concentration in cotyledons which had been exposed to elicitor for various time intervals was

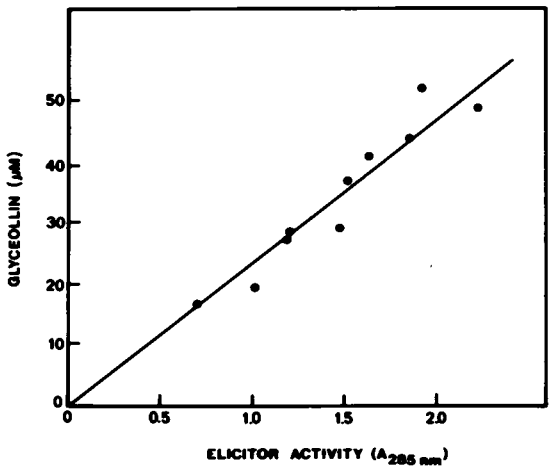

FIG. 1. Correlation between A_{285} and glyceollin content of wound-droplet solutions. The same amount of elicitor (ranging from 0.05–0.8 μg carbohydrate/cotyledon) was applied, in 100 μl of 10 mM sodium phosphate, pH 7.2, containing 100 μg/ml gentamicin, to each of a set of 10 wounded cotyledons. The treated cotyledons were incubated at 100% humidity for 20 hr at 26 C. The droplets retained on the wound surfaces of each set of cotyledons were rinsed into 20 ml of H_2O, and the resulting wound-droplet solutions were assayed for their A_{285} and for their glyceollin content. The A_{285} (0.2) and glyceollin content (3 μM) of a set of cotyledons treated only with the solution of gentamicin were subtracted from each datum.

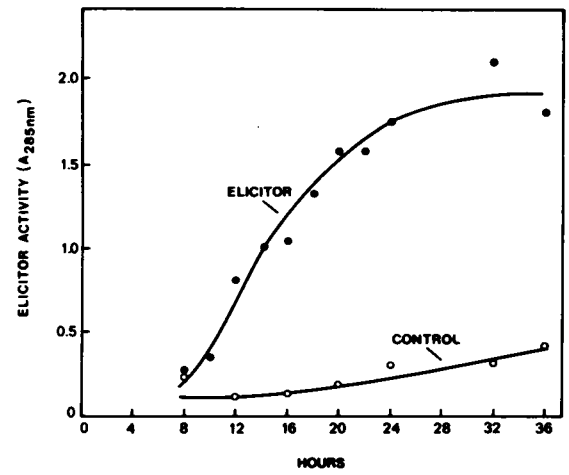

FIG. 2. Rate of glyceollin accumulation in elicitor-treated cotyledons as measured by A_{285} of wound-droplet solutions. The A_{285} of wound-droplet solutions from sets of 10 cotyledons treated with elicitor (0.1 μg carbohydrate/cotyledon) was measured at various intervals after elicitor application. The cotyledons were incubated at 100% humidity at 26 C. The response of cotyledons treated with elicitor (●) was compared to the response of cotyledons treated with H_2O (○). The antibiotic gentamicin (100 μg/ml) was present in all solutions applied to cotyledons. Each point represents the average for three sets of cotyledons.

determined by measuring the A_{285} of wound-droplet solutions (Fig. 2). Ten hr after the application of 0.1 μg of elicitor to each set of cotyledons, the A_{285} value of wound-droplet solutions rises significantly above that obtained for cotyledons which had been treated with H_2O rather than elicitor. After approximately 20 hr of incubation, the A_{285} value stops increasing and remains constant for up to 36 hr. The background A_{285} value of water-treated control cotyledons remains essentially constant for 16 hr and then slowly increases. An incubation period of 20 hr was selected for elicitor assays in order to minimize the contribution of the background absorbance and to maximize elicitor response.

The actual glyceollin content of wound-droplet solutions was also determined after various periods of incubating cotyledons with elicitor. A comparison of the accumulation of glyceollin with the A_{285} of wound-droplet solutions is presented in Figure 3. The data in this figure demonstrate that there is an increase in A_{285} which precedes the accumulation of glyceollin. Also, the rise in glyceollin content continues even though the A_{285} has reached a plateau. Therefore, the ratio of the glyceollin concentrations to A_{285} changes throughout the incubation of wounded cotyledons with elicitor. The ratio is constant for various amounts of elicitor incubated for a specific period of time (Fig. 1).

Effect of Varying Amount of Elicitor Applied to Cotyledons. Amounts of Pms elicitor in the range of 0.008 to 1 μg/cotyledon were applied to wounded cotyledons and the accumulation of glyceollin was measured (Fig. 4). The minimum amount of elicitor giving a detectable response was approximately 0.02 μg/cotyledon. The A_{285} value of the wound-droplet solutions increased with increasing amounts of elicitor up to approximately 0.5 μg/cotyledon.

Hypocotyl Assay of Elicitor Activity. The hypocotyls of intact soybean seedlings were used in a second biological assay of elicitor activity. The assay described here is a refined, quantitative form of the semiquantitative assay used by Klarman and Gerdemann (21) and by Keen (17).

Five-day-old soybean seedlings were removed from their soil and rinsed in tap H_2O to eliminate clinging potting material. The intact seedlings were then mounted on horizontal stainless steel needles (1 mm diameter, 11 cm length) by piercing the hypocotyls 5 mm below the cotyledons. The resulting vertical slit wounds

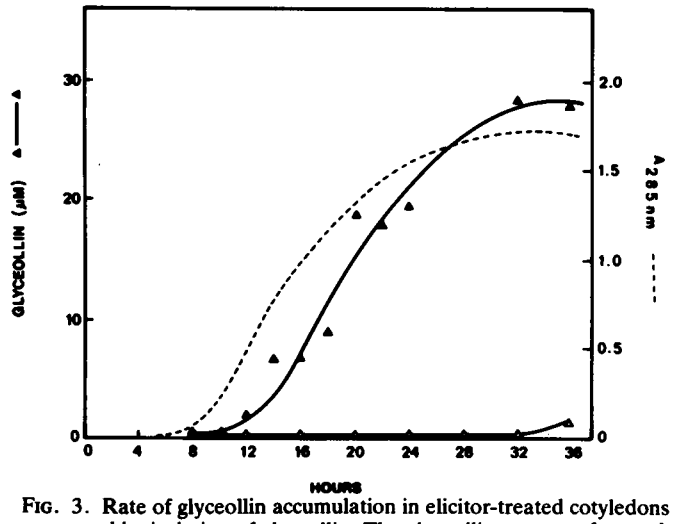

FIG. 3. Rate of glyceollin accumulation in elicitor-treated cotyledons as measured by isolation of glyceollin. The glyceollin content of wound-droplet solutions from sets of 10 cotyledons treated with elicitor (0.1 μg carbohydrate/cotyledon) was determined at various intervals after elicitor application. The cotyledons were incubated in 100% humidity at 26 C. Glyceollin was measured using a TLC method as described in the text. The glyceollin content of wound-droplet solutions from cotyledons treated with elicitor (▲) and H_2O (△) was compared to the A_{285} of these solutions (---). The antibiotic gentamicin (100 μg/ml) was present in all solutions applied to cotyledons.

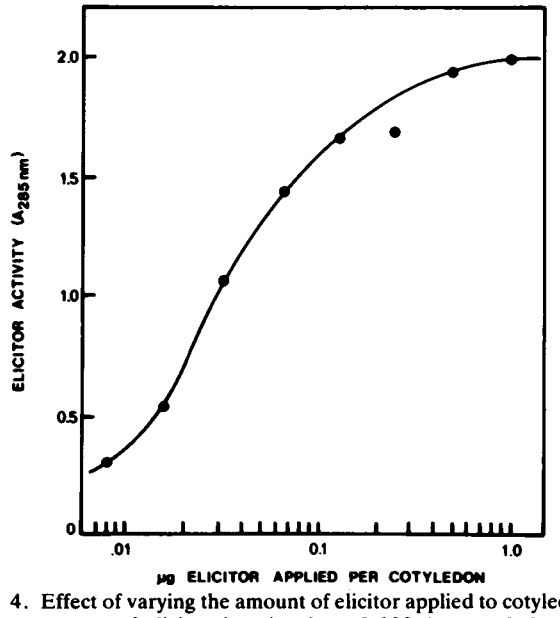

FIG. 4. Effect of varying the amount of elicitor applied to cotyledons. The same amount of elicitor (ranging from 0.008–1 μg carbohydrate/cotyledon) was applied to each of a set of 10 wounded cotyledons. The treated cotyledons were incubated at 100% humidity for 20 hr at 26 C and the A_{285} of the wound-droplet solution from each set of 10 cotyledons was measured. The A_{285} of the wound-droplet solutions of cotyledons treated with H_2O (0.2) was subtracted from each datum. The antibiotic gentamicin sulfate (100 μg/ml) was present in all solutions applied to cotyledons. Each point represents the average of three sets of cotyledons.

in the hypocotyls were about 5 mm in length. A set of 10 seedlings was mounted on each needle, and the seedlings were suspended with their roots in H_2O to within 2 cm of the wounds. A 20-μl drop of an elicitor preparation in a solution of 100 μg/ml gentamicin sulfate was applied to each hypocotyl wound. Care was taken to prevent the wounded surfaces from becoming dry prior to application of the sample.

Each set of 10 seedlings was removed from its needle after incubation in a closed chamber at 26 C for 24 hr in 100% humidity. A 14-mm long segment of hypocotyl, centered on the wound, was cut from each seedling, and the set of wound segments was dried in a stream of warm air to remove surface moisture. Each set of segments was then homogenized in 15 ml of 95% ethanol for 1 min at maximum speed in a Sorval Omni-Mixer. Insoluble material was removed from the suspension by passage through a GF/A filter (Whatman) and the resulting solution was dried in a stream of filtered air at 50 C. The dry residue was dissolved in 200 μl of ethyl acetate and a 100-μl aliquot from each sample was applied to a portion of a preparative thin layer plate of silica gel. Each 20 × 20 cm plate contained four samples and a glyceollin standard. The plates were developed with hexane-ethyl acetate-methanol (60:40:5, v/v). Glyceollin in the samples was detected by UV absorbance (R_F = 0.32) and identified by comparison to the glyceollin standard. The glyceollin content of each sample from a set of 10 treated hypocotyls was determined by extracting the glyceollin from the silica with 5 ml of 95% ethanol and measuring the absorbance of the extract at 285 nm.

The biological activity of compounds identified on TLC plates as glyceollin was tested periodically by the ability of the compound to inhibit the growth of *C. cucumerinum* in a combined TLC-fungitoxicity assay (20).

Rate of Glyceollin Accumulation in Hypocotyls Treated with Pms Elicitor. Hypocotyls were treated with 0.6 μg of Pms elicitor each, and the glyceollin content was determined, as described above, after various time intervals (Fig. 5). An increase in glyceollin content was first detected 8 hr after application of the elicitor. The glyceollin content increased until approximately 24 hr of incubation.

The concentration of glyceollin required to decrease the rate of growth of Pms by 50% (ED_{50}) has been reported to be 70 μM (20). This concentration corresponds to 3.5 nmoles of glyceollin/

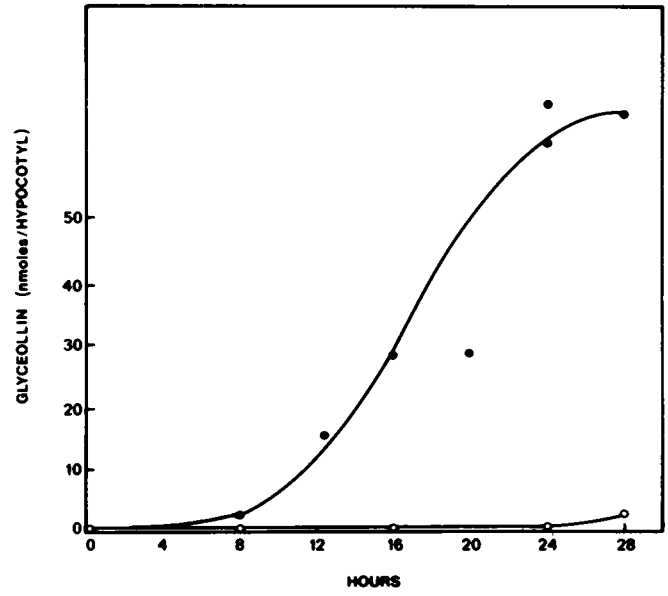

FIG. 5. Rate of accumulation of glyceollin in elicitor-treated hypocotyls. Elicitor (0.6 μg carbohydrate/hypocotyl) was applied, in 20 μl of 10 mM sodium phosphate, pH 7.2, containing 100 μg/ml of gentamicin sulfate, to each of a set of 10 wounded hypocotyls of soybean seedlings. The treated seedlings were incubated at 100% humidity for 24 hr in the dark at 26 C. Glyceollin was extracted from the wound regions of each set of seedlings and separated from other compounds by TLC. The glyceollin was then quantitated by the absorbance of the purified glyceollin solution at 285 nm. The accumulation of glyceollin in elicitor-treated hypocotyls (●) was compared to water-treated hypocotyls (○).

hypocotyl. As the data in Figure 5 indicate, the ED_{50} concentration for the entire hypocotyl segment is reached within the first 12 hr of incubating the hypocotyl with elicitor.

Effect of Varying Amounts of Elicitor Applied to Hypocotyls. The response of the soybean hypocotyls to varying amounts of Pms elicitor was determined by treating hypocotyls with elicitor (0.1–5 μg/hypocotyl) and measuring the glyceollin content of the hypocotyls after 24 hr of incubation at 26 C (Fig. 6). The hypocotyls accumulated detectable quantities of glyceollin in response to amounts of elicitor as low as 0.1 μg/hypocotyl. The hypocotyls produced a maximum amount of glyceollin in response to 1 μg/hypocotyl.

Glyceollin Accumulation in Soybean Cell Suspension Cultures Stimulated by Pms Elicitor. The application of elicitor to organs of soybean seedlings requires the disruption of the cuticle and of the external cell layers to permit the elicitor to interact effectively with the plant cells. The trauma associated with wounding can be avoided by the use of soybean cell suspension cultures in place of plant organs. An elicitor assay has been developed (11) using soybean cell suspension cultures in order to demonstrate that the response to elicitor does not require mechanical wounding. The increased glyceollin content is detectable approximately 12 hr after addition of elicitor. The level of glyceollin in both the cells and medium continues to increase for at least 36 hr.

Isolation of Extracellular Elicitor Present in Cultures of Pms. Pms race 1 was grown on liquid medium for 14 days and the cultures were filtered (GF/A, Whatman) to remove the mycelia. The culture filtrates were concentrated 100 times by rotary evaporation under reduced pressure at 40 C and dialyzed against two changes (2 liters each) of deionized H_2O. Dialysis divided the filtrate into "small extracellular elicitor" and "large extracellular elicitor" based on the ability of the molecules to pass through or to be retained by the dialysis membrane, respectively.

Purification of Large Extracellular Elicitor. The stability of the large extracellular elicitor preparations to pH extremes, heat, and pronase was measured using the cotyledon assay. The large extracellular elicitor did not lose activity when incubated in a pH range from 2 to 10.2 at 60 C for 1 hr. This preparation was also stable to pronase treatment (1 mg/ml pronase [Sigma]. 37 C, 12 hr in 10 mM sodium phosphate, pH 7.2), and no activity was lost upon exposure at pH 7 to 121 C for 3 hr. The stability of the elicitor under these conditions indicates that the elicitor is unlikely to be a protein.

The ionic characteristics of the large extracellular elicitor were investigated by determining the affinity of the elicitor for anion and cation exchange materials at various hydrogen ion concentrations. This elicitor has no affinity for the anion exchangers diethylaminoethyl- or quaternary aminoethyl-Sephadex (Pharmacia) at pH values of 8 and 9, respectively. The elicitor also has no affinity for the cation exchangers carboxymethyl- or sulfopropyl-Sephadex (Pharmacia) at pH 5 and 4, respectively, nor did the elicitor bind to the strong cation exchange resin AG 50W (analytical grade Dowex 50W, Bio-Rad) at pH 2. The majority of the protein present in the extracellular media was separated from the elicitor by several of these ion exchange materials but both the elicitor and the majority of the polysaccharide present in the extracellular media of Pms cultures did not bind to any of these materials under the specific conditions. These procedures also rule out the possibility that the elicitor is a nucleic acid or another highly charged molecule.

The elicitor does, however, bind to the strong anion exchange resin AG 1-X1 (analytical grade Dowex 1-X1, Bio-Rad) at pH 10.2. The elicitor's lack of affinity for DEAE-cellulose, the binding of the elicitor to AG 1-X1, and molecular sieving chromatography form the basis of the purification of this elicitor.

The first step in the purification scheme of large extracellular elicitor is passage through DEAE-cellulose. This step was designed to reduce the amount of protein present in the preparation in order to enhance subsequent chromatography on AG 1-X1. Large extracellular elicitor preparations containing approximately 200 mg of protein and 200 mg of carbohydrate (the yield from 2 liters of Pms culture filtrate) were applied in 100 ml of 10 mM sodium phosphate, pH 8, to a column (1.8 × 25 cm) of DEAE-cellulose (DE52, Whatman) equilibrated with 10 mM sodium phosphate, pH 8. The column was eluted with 250 ml of 10 mM sodium phosphate, pH 8, followed by elution with a linear 10 to 500 mM sodium phosphate, pH 8, gradient (total gradient volume 500 ml). The column effluent was assayed for carbohydrate and protein (Fig. 7). Elicitor activity was only detected associated with compounds which have no affinity for this anion exchange material.

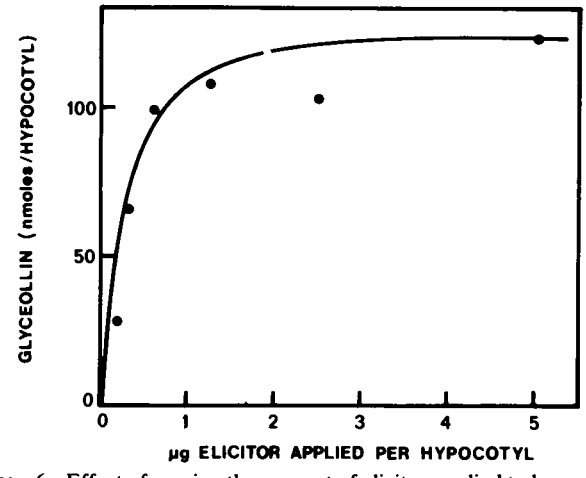

FIG. 6. Effect of varying the amount of elicitor applied to hypocotyls. The same amount of elicitor (ranging from 0.2–5 μg carbohydrate/hypocotyl) was applied to each of a set of 10 hypocotyls. The treated seedlings were incubated for 24 hr at 100% humidity at 26 C. Glyceollin was measured using the TLC method described in the text. The glyceollin content of hypocotyls treated with H_2O (3 moles/hypocotyl) was subtracted from each datum. The antibiotic gentamicin (100 μg/ml) was present in all solutions applied to hypocotyls.

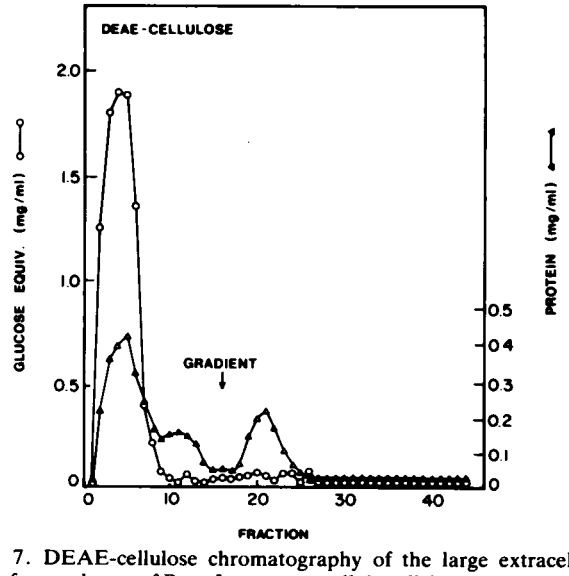

FIG. 7. DEAE-cellulose chromatography of the large extracellular elicitor from cultures of Pms. Large extracellular elicitor was applied to a DEAE-cellulose column (1.8 × 25 cm) equilibrated with 10 mM sodium phosphate, pH 8. The column was eluted with 250 ml of this buffer followed by a linear 10 to 500 mM gradient in buffer concentration (total volume of gradient was 500 ml). The 20-ml fractions were assayed for protein (△) and carbohydrate (○). All of the elicitor activity passed directly through the column (fractions 2–7).

The fractions from the DEAE-cellulose column which had elicitor activity (fractions 2–7) were concentrated to 20 ml, dialyzed, adjusted to 10 mM in glycine/NaOH buffer, pH 10.2, and applied to a column (20 ml) of AG 1-X1 (50 mesh, Bio-Rad) equilibrated with the same buffer. Essentially all (95%) of the carbohydrate and elicitor activity was bound to the column and was eluted together as a single peak in the middle of a linear 10 to 200 mM buffer gradient (total gradient volume 200 ml). The protein present in these samples remained bound to the AG 1-X1 resin and was not detected in any of the fractions collected. Thus, chromatography on AG 1-X1 at pH 10.2 effectively removed from the elicitor preparation all measurable protein and other anionic molecules which did not bind the DEAE-cellulose.

The elicitor was then fractionated on the basis of size by molecular sieving chromatography. The fractions released from the AG 1-X1 column, which contained elicitor activity, were combined, dialyzed, concentrated to 2 ml, and applied to a column (2.3 × 71 cm) of Sephadex G-100 (140–200 mesh, Pharmacia). The column was eluted with 100 mM sodium phosphate, pH 7.2. Elicitor activity was found throughout the column effluent associated with a heterogeneous distribution of polysaccharides (Fig. 8). These polysaccharides vary in size from 10,000 to greater than 100,000 in mol wt. This mol wt estimate is based on the fractionation range of dextrans on Sephadex G-100. The effluent from the Sephadex G-100 column was divided into two fractions. Fraction A (column fractions 14–22) contained elicitor molecules with mol wt greater than approximately 40,000. Fraction B (column fractions 16–30) contained elicitor molecules with mol wt of approximately 10,000. Figure 8 demonstrates that the polysaccharides in fraction A have a higher elicitor activity/unit of carbohydrate than the polysaccharides in fraction B.

Purification of Small Extracellular Elicitor. The small extracellular elicitor is that material which passed through the dialysis membrane used to divide the extracellular elicitor into its large and small components. The small elicitor was diluted by this procedure into the deionized H_2O (4 liters) against which the extracellular culture fluid was dialyzed. This solution was concentrated to 40 ml by rotary evaporation under reduced pressure at 40 C and the elicitor present in this solution was separated from salts and the other small molecules by molecular sieving chromatography on a column (3.2 × 50 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad). The column was eluted with H_2O. The void fractions, which contained the elicitor activity, were combined and evaporated to dryness. The dry elicitor preparation was dissolved in 15 ml of 10 mM glycine/NaOH buffer, pH 10.2, and applied to a column (1.5 × 16 cm) of AG 1-X1 (50 mesh, Bio-Rad) that had been equilibrated with the 10 mM glycine/NaOH buffer. The column was washed with the equilibration buffer and then eluted with a linear 0 to 300 mM gradient of NaCl prepared in the same buffer. The column fractions were assayed for carbohydrate and for elicitor activity (Fig. 9). A peak in elicitor activity corresponding to a peak in carbohydrate content eluted between 140 and 170 mM NaCl.

The active fractions (47–57) from the AG 1-X1 column were combined, concentrated to 20 ml, desalted by molecular sieving chromatography on Bio-Gel P-2 (Bio-Rad) and applied to a column (2.2 × 78 cm) of Sephadex G-50 (medium, Pharmacia). This molecular sieving column was eluted with 100 mM sodium phosphate, pH 7.2. The fractions containing the elicitor activity were not the fractions richest in carbohydrate (Fig. 10). The fractions (24–30) containing elicitor activity were combined, concentrated to 5 ml and rechromatographed under the same conditions on the Sephadex G-50 column. As expected, after rechromatography on Sephadex G-50, the elicitor activity of the column effluent roughly coincided with carbohydrate content (Fig. 11). The approximate mol wt of the elicitor, as estimated from the fractionation range of dextrans on Sephadex G-50, is 2,500. The amount of the inactive carbohydrate material still present in this preparation could not be determined. No further purification of this fraction was attempted.

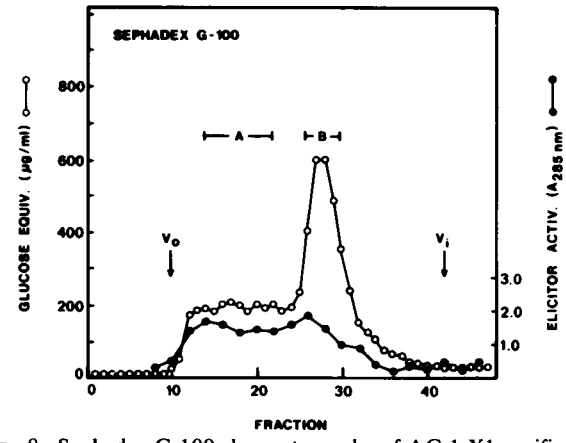

FIG. 8. Sephadex G-100 chromatography of AG 1-X1 purified large extracellular elicitor from cultures of Pms. Elicitor purified by AG 1-X1 chromatography was applied to a column (2.2 × 71 cm) of Sephadex G-100 and the column was eluted with 100 mM sodium phosphate, pH 7.2. Fractions were 6 ml. The void volume (V_o) was 72 ml and the inclusion volume (V_i) was 250 ml. Carbohydrate (○) in glucose equivalents was measured by the anthrone procedure. Elicitor activity (●) was measured by the cotyledon assay using the equivalent of 5 μl of each column fraction/cotyledon. The elicitor was separated by this technique into two components, Fraction A (column fractions 14–22) and fraction B (column fractions 26–30).

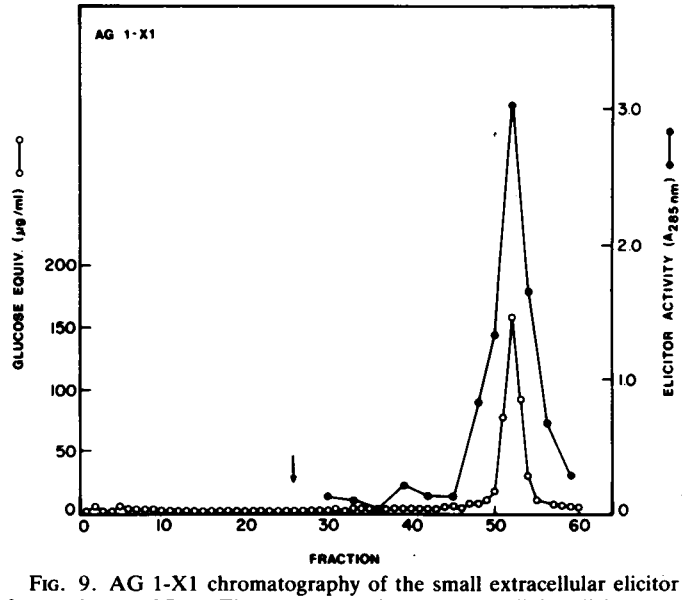

FIG. 9. AG 1-X1 chromatography of the small extracellular elicitor from cultures of Pms. The concentrated small extracellular elicitor was applied in 15 ml to a column (1.5 × 16 cm) of AG 1-X1 equilibrated with 10 mM glycine/NaOH buffer, pH 10.2. The column was washed with 100 ml of the glycine/NaOH buffer. The arrow indicates the start of a gradient of increasing NaCl concentration from 0 to 300 mM in a total of 160 ml. Fractions were 5 ml in volume. The elicitor assay (●) was carried out by applying to each cotyledon the equivalent of 2 μl of each fraction. Carbohydrate (○) was measured by the anthrone procedure. The peak of the elicitor activity and the carbohydrate eluted at a concentration of 0.16 M NaCl.

Composition and Structure of Extracellular Elicitor of Pms. The neutral sugar compositions of the purified small extracellular elicitor and fractions A and B of the purified large extracellular elicitor were determined by gas chromatographic analysis of

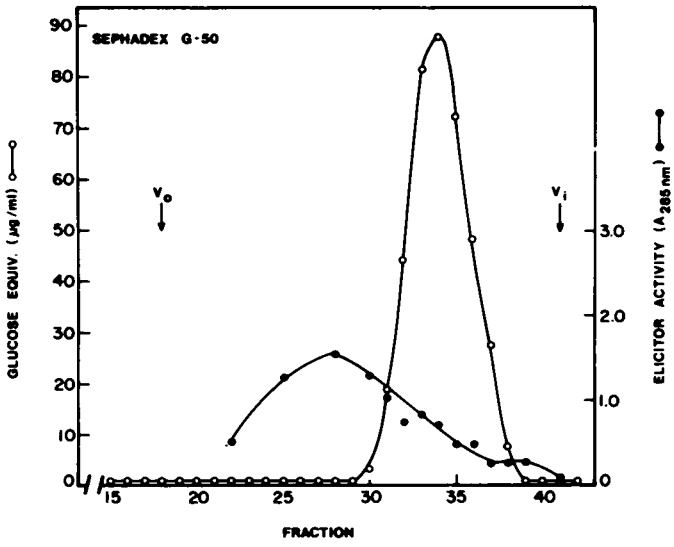

FIG. 10. Sephadex G-50 chromatography of AG 1-X1 purified small extracellular elicitor. Small extracellular elicitor purified by AG 1-X1 was applied to a column (2.2 × 78 cm) of Sephadex G-50 eluted with 100 mM sodium phosphate, pH 7.2. The void volume for this column (V_0) was 130 ml and the inclusion volume (V_i) was 300 ml. Fractions were 7.4 ml in volume. The carbohydrate content (O) of each fraction was determined in glucose equivalents by the anthrone procedure. Elicitor activity (●) was determined by the cotyledon assay using the equivalent of 10 μl of sample/cotyledon. The elicitor in fractions 24 to 30 was combined and concentrated for rechromatography on Sephadex G-50.

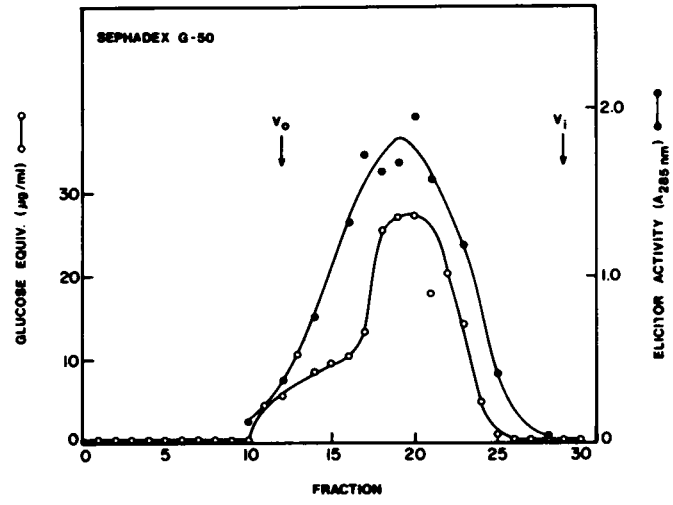

FIG. 11. Rechromatography of small extracellular elicitor on Sephadex G-50. The column fractions containing elicitor activity from four Sephadex G-50 chromatography runs, as described in Fig. 10, were combined, concentrated and reapplied to the Sephadex G-50 column. The conditions for rechromatography and assay were the same as described in Fig. 10, except that the fraction volume was 10 ml.

the alditol acetate derivatives of these polysaccharides (16). In all three cases, glucose made up at least 95% of the neutral sugars with mannose and galactose present in minor amounts (less than 1%).

The glycosyl residues of a polysaccharide can be linked to neighboring glycosyl residues through several of their carbon atoms. The partially methylated alditol acetate derivatives of a polysaccharide retain the information on which carbon atoms of each glycosyl residue have neighboring glycosyl residues linked to them. The partially methylated alditol acetate derivatives of the extracellular elicitor were synthesized (5). These derivatives were separated by GLC and were identified by their retention times relative to known standards and by mass spectrometry of the effluent from the gas chromatograph. Gas chromatograms of the partially methylated alditol acetate derivatives of large extracellular elicitor Fractions A and B, and of the mycelial walls (4) of Pms are presented in Figure 12. The glycosyl-linkage composition of large extracellular elicitor Fractions A and B, and of the mycelial walls of Pms are presented in Table I in terms of the percentage of total glycosyl residues present in each preparation. Glucosyl residues which are 3-linked predominate in each of these samples. The percentage of terminal glucosyl residues closely approximates the percentage of the branched 3,6-linked glucosyl residues in each sample. This equivalence is consistent with the general structural requirement that each terminal glycosyl residue of a polysaccharide be glycosidically linked either directly or through intervening glycosyl residues to a branched glycosyl residue.

The mycelial walls of Pms contain in addition to the terminal, 3-linked and 3,6-linked glucosyl residues, another quantitatively important glycosyl component not present in the elicitor, namely

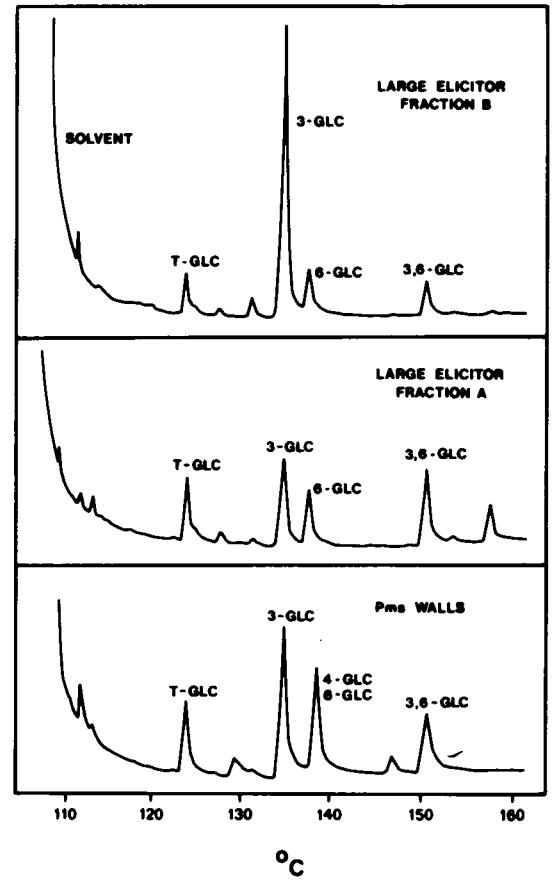

FIG. 12. Gas chromatograms of the partially methylated alditol acetates derived from the purified mycelial walls of Pms and from the large extracellular elicitor fractions A and B. The gas chromatographic separations were performed on a Hewlett Packard model 7620 A gas chromatograph using a 120 × 0.3 cm (o.d.) copper column containing a mixture of 0.2% poly (ethylene glycol adipate), 0.2% poly (ethylene glycol succinate), and 0.4% XF-1150 on Gas Chrom P (100–120 mesh). Chromatography was performed using a helium flow rate of approximately 6 ml/min. Gas chromatograph electrometer settings were range 1 attenuation 2. The 1- to 3-μl sample was injected at a column temperature of 110 C, and after 2 min the temperature was raised at 1 C/min to 190 C. The linkages to each sugar derivative are indicated by numerical prefixes: thus, 3,6-Glc indicates that other glycosyl residues are glycosidically linked in the polysaccharides to the 3 and the 6 carbons of these glucosyl residues. Terminal residues are indicated by T- (*e.g.* T-Glc).

Table I. *Glycosyl Linkage Composition of Pms Mycelial Walls and of Large Extracellular Elicitor Fractions A and B*

The glycosidic linkages to each sugar derivative are indicated by numerical prefixes: 3,6-Glc indicates that other glycosyl residues are glycosidically linked in the polysaccharides to the 3 and 6 carbons of these glucosyl residues. Terminal residues are indicated by T- (*e.g.* T-Glc). The figures presented are the mole per cent of each glycosyl residue found in the polysaccharides.

	Glycosyl Linkages				
	T-Glc	3-Glc	4-Glc	6-Glc	3,6-Glc
	% total glycosyl residues				
Pms mycelial walls	15	39		28	18
Large elicitor A	24	32	0	18	27
Large elicitor B	8	72	0	12	9

4-linked glycosyl residues. Mass spectral analysis of the fraction of the effluent of the gas chromatograph which contains the derivative corresponding to 4-linked glucosyl residues of the mycelial walls indicates that the derivative corresponding to 6-linked glucosyl residues is also present. Large extracellular elicitor fractions A and B contain 6-linked glucosyl residues, but no 4-linked glucosyl residues were detected in these preparations.

The degree of branching in each sample is proportional to its content of 3,6-linked glucosyl residues. The data in Table I show that fraction A elicitor is more highly branched than the elicitor of fraction B and that the Pms mycelial walls are intermediate in degree of branching. The more branched fraction A elicitor is more active than fraction B elicitor.

The small extracellular elicitor, although possibly contaminated with some inactive 3-linked glucan, has the chemical properties and a structure similar to the large extracellular elicitor.

Elicitor Activity of Other Polysaccharides, Glycosides, and Sugars. The finding that the extracellular elicitors are polysaccharides prompted tests to determine whether other polysaccharides, glycosides, and sugars have elicitor activity. None of the compounds tested, with the exception of laminarin, exhibited elicitor activity in the cotyledon or hypocotyl assays. Laminarin, a mixture of β-1,3-linked glucans obtained from the alga *Laminaria hyperborea* (27), has a low but significant activity in the cotyledon assay but not in the hypocotyl assay. Twenty μg of laminarin have the same elicitor activity as 0.01 μg of Pms elicitor. The other polysaccharides (yeast mannan, larch galactan, and nigeran), glycosides (lactose, maltose, cellobiose, α-methyl-D-mannopyranoside, α-methyl-D-galactopyranoside, α-methyl-D-glucopyranoside, β-methyl-D-glucopyranoside), and sugars (mannose, galactose, and glucose) tested failed to stimulate glyceollin accumulation in soybean tissue even at 20 μg/cotyledon and 4 μg/hypocotyl.

DISCUSSION

Cotyledons and hypocotyls of soybean seedlings respond to Pms elicitor by the accumulation of the soybean phytoalexin glyceollin. As little as 0.02 μg of Pms elicitor is sufficient to stimulate glyceollin accumulation in a cotyledon and 0.1 μg of elicitor is sufficient for the stimulation of glyceollin accumulation in a hypocotyl. The average mol wt of the Pms elicitor is approximately 100,000 (5). A soybean cotyledon, therefore, responds to about 10^{-12} moles of elicitor. This high sensitivity of soybean cells to the Pms elicitor suggests that soybean cells have receptor molecules which specifically bind elicitor molecules and that elicitors have a specific biological function. Indeed, within 12 hr, the elicitor stimulated accumulation of glyceollin had reached the ED_{50} concentration for Pms in a cotyledon or a hypocotyl treated with less than 1 μg of the Pms elicitor.

A comparison of the A_{285} and glyceollin content (Fig. 3) indicates that materials absorbing at 285 nm are present in the wound-droplets before glyceollin is detected and also that the ratio of glyceollin to other materials absorbing at 285 nm increases with the time of incubation. These observations are consistent with the idea that glyceollin is found in the wound-droplets only after precursors to glyceollin have accumulated. These precursors are also likely to absorb at 285 nm and may account for the fact that the A_{285} of these solutions is observed to increase prior to an increase in glyceollin content.

The striking feature of the three biological assays for elicitor activity is that the rate of glyceollin accumulation in the assays is so similar (Figs. 2 and 6 and ref. 11). In all three cases, glyceollin can be first detected 10 to 12 hr after treatment with elicitor and, subsequently, the level of glyceollin continues to increase for at least an additional 10 hr. Results to be presented (11) also demonstrate that the stimulation of glyceollin accumulation is not linked to wounding of the soybean tissues, since soybean cell suspension cultures exhibit the same response as the tissues of soybean seedlings. We concluded that the ability of the Pms elicitor to stimulate the accumulation of glyceollin is a general characteristic of soybean cells.

The ability of the Pms elicitor to resist pH extremes, heat, and pronase, the size heterogeneity of the elicitor, and the fact that proteins, nucleic acids, and other charged molecules have been eliminated by the purification techniques used, indicate that the extracellular elicitors from Pms cultures are polysaccharides. The structural investigations (Table I) of the large extracellular elicitor indicate that it is predominantly a 1,3-linked glucan.

Fraction A of the large extracellular elicitor has a higher elicitor activity per unit of carbohydrate than does fraction B (Fig. 8). The data from methylation analysis (Table I) indicate that fraction A of the large extracellular elicitor is more highly branched than fraction B; the higher degree of branching may be correlated with higher relative elicitor activity. The correlation between degree of branching and biological activity of the elicitor suggests that the terminal glycosyl residues linked to the branch points are involved in elicitor activity.

Zevenhuizen and Bartnicki-Garcia (38) determined that the mycelial walls of *Phytophthora cinnamomi* are composed of 5% cellulose and 55% base-insoluble glucan. Their results indicated that the base-insoluble glucan contains β-1,3-linked glucosyl residues (58%) with terminal glucosyl residues (15.4%) connected to 3,6-linked glucosyl residues (20.7%). The methylation analysis of Pms walls presented here (Table I) confirms those findings. The 4-linked glucosyl residues found in the present work were most probably derived from the β-1,4-linked glucosyl residues of cellulose, a known polymeric constituent of the mycelial walls of *Phytophthora* sp. (38).

The only commercially available compound tested which had elicitor activity, and only in the cotyledon assay, was laminarin. Commercial laminarin is a mixture of glucans isolated from *Laminaria hyperborea* (27). The insoluble portion of laminarin is an unbranched β-1,3-linked glucan with approximately 25 glucosyl residues per molecule (27). Laminarin also contains a β-1,3-linked glucan with a single terminal glucosyl residue linked at the six position to one of the 3-linked glucosyl residues in each molecule (27). These sparsely branched glucans may be responsible for the low elicitor activity of commercial laminarin.

The most significant finding derived from the purification and characterization of the extracellular elicitor of Pms is that both the large and the small elicitors are similar in composition and structure to the base-insoluble glucan component of Pms walls (38). The elicitor and the base-insoluble glucan are both 3-linked glucans with branches originating at 3,6-linked glucosyl residues. This fact suggests that the mycelial walls of Pms may be the source of extracellular elicitor. The similarity in composition and structure of the large and small extracellular elicitors also suggests that there is but a single extracellular elicitor which is

very heterogeneous in size and in the relative abundance of branched residues. These glucans account for all of the elicitor activity of the extracellular fluid of Pms cultures (17, 19).

The sensitivity of soybean cells to low levels of Pms elicitor, the structural relatedness of Pms elicitor to the base-insoluble component of Pms walls, and the availability of elicitor in culture filtrates all indicate that this glucan elicitor is of biological significance in host-pathogen interactions and that the glucan triggers phytoalexin accumulation in soybeans infected with Pms.

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LITERATURE CITED

1. Anderson, A. and P. Albersheim. 1973. Induction of phaseollin and browning in *Phaseolus vulgaris* tissue by macromolecules secreted by *Colletotrichum lindemuthianum*. Second Int. Congr. Plant Pathol., Phytopathol. Abstr. 0764.
2. Anderson-Prouty, A. and P. Albersheim. 1975. Host-pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogen's host. Plant Physiol. 56: 286–291.
3. Ayers, A. R., J. Ebel, and P. Albersheim. 1974. A highly potent elicitor of hydroxyphaseollin synthesis in soybean tissues has been purified from the culture filtrates of *Phytophthora megasperma* var. *sojae*. Proc. 66th Annu. Mtg. Am. Phytophathol. Soc., August 17.
4. Ayers, A. R., J. Ebel, B. Valent, and P. Albersheim. 1976. Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. Plant Physiol. 57: 760–765.
5. Ayers, A. R., B. Valent, J. Ebel, and P. Albersheim. 1976. Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol. 57: 766–774.
6. Burden, R. S. and J. A. Bailey. 1975. Structure of the phytoalexin from soybean. Phytochemistry 14: 1389–1390.
7. Cruickshank, I. A. M. and D. R. Perrin. 1963. Studies on phytoalexins. VI. Pisatin: the effect of some factors on its formation in *Pisum sativum* L. and the significance of pisatin in disease resistance. Aust. J. Biol. Sci. 16: 111–128.
8. Cruickshank, I. A. M. and D. R. Perrin. 1968. The isolation and partial characterization of monilicolin A, a polypeptide with phaseollin inducing activity from *Monilinia fructicola*. Life Sci. 7: 449–458.
9. Day, P. R. 1973. *In:* A. Kelman and L. Sequeira, eds., Genetics of Host-Parasite Interactions. W. H. Freeman and Co., San Francisco.
10. Dische, Z. 1962. General color reactions. *In:* R. L. Whistler and M. L. Wolfrom, eds., Methods in Carbohydrate Chemistry, Vol. I. Academic Press, New York. pp. 478–512.
11. Ebel, J., A. R. Ayers, and P. Albersheim. 1976. Host-pathogen interactions. XII. The response of suspension-cultured soybean cells to the elicitor isolated from *Phytophthora megasperma* var. *sojae*, a fungal pathogen of soybeans. Plant Physiol. 57: 775–779.
12. Frank, J. A. and J. D. Paxton. 1970. A fungal inducer of soybean phytoalexin from *Phytophthora megasperma* var. *sojae*. Phytopathology 60: 1292.
13. Frank, J. A. and J. D. Paxton. 1971. An inducer of soybean phytoalexin and its role in the resistance of soybeans to *Phytophthora* rot. Phytopathology 61: 954–956.
14. Hadwiger, L. A. 1972. Phytoalexin production induced by heavy metals—proposed mode of action. Phytopathology 62: 761.
15. Hadwiger, L. A. and M. E. Schwochau. 1971. Specificity of DNA intercalating compounds in the control of phenylalanine ammonia lyase and pisatin levels. Plant Physiol. 47: 346–351.
16. Jones, T. M. and P. Albersheim. 1972. A gas-chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. Plant Physiol. 49: 926–936.
17. Keen, N. T. 1974. Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187: 74–75.
18. Keen, N. T. 1975. The isolation of phytoalexins from germinating seeds of *Cicer arietinum*, *Vigna sinensis*, *Arachis hypogaea* and other plants. Phytopathology 65: 91–92.
19. Keen, N. T., J. E. Partridge, and A. I. Zaki. 1972. Pathogen-produced elicitor of a chemical defense mechanism in soybeans monogenetically resistant to *Phytophthora megasperma* var. *sojae*. Phytopathology 62: 768.
20. Keen, N. T., J. J. Sims, D. C. Erwin, E. Rice, and J. E. Partridge. 1971. 6a-Hydroxyphaseollin: an antifungal chemical induced in soybean hypocotyls by *Phytophthora megasperma* var. *sojae*. Phytopathology 61: 1084–1089.
21. Klarman, W. L. and J. W. Gerdemann. 1963. Induced susceptibility in soybean plants genetically resistant to *Phytophthora sojae*. Phytopathology 53: 863–864.
22. Klarman, W. L. and J. W. Gerdemann. 1963. Resistance of soybeans to three phytophthora species due to the production of a phytoalexin. Phytopathology 53: 1317–1320.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
24. Lwoff, A., L. Siminovitch, and N. Kjeldgaard. 1950. Induction de la lyse bactériophagique de la totalité d'une population microbienne lysogène. Compt. Rend. Acad. Sci. 231: 190.
25. Mansfield, J. W., J. A. Hargreaves, and F. C. Boyle. 1974. Phytoalexin production by live cells in broad bean leaves infected with *Botrytis cinerea*. Nature 252: 316–317.
26. McKeen, W. E. and P. K. Bhattacharya. 1970. Limitation of infection by *Erysiphe graminis* f. sp. *hordei* culture CR3 by the algerian gene Mla in barley. Can. J. Bot. 48: 1109–1113.
27. Nelson, T. E. and B. A. Lewis. 1974. Separation and characterization of the soluble and insoluble components of insoluble laminarin. Carbohyd. Res. 33: 63–74.
28. Paxton, J. D. 1971. Inducer of soybean phytoalexin. Phytopathology 61: 1025.
29. Paxton, J. D., D. J. Goodchild, and I. A. M. Cruickshank. 1974. Phaseollin production by live bean endocarp. Physiol. Plant Pathol. 4: 167–171.
30. Rathmell, W. G. and D. S. Bendall. 1971. Phenolic compounds in relation to phytoalexin biosynthesis in hypocotyls of *Phaseolus vulgaris*. Physiol. Plant Pathol. 1: 351–362.
31. Schwochau, M. E. and C. A. Hadwiger. 1968. Stimulation of Pisatin production in *Pisum sativum* by actinomycin D and other compounds. Arch. Biochem. Biophys. 126: 731–733.
32. Tomiyama, K. 1966. Double infection by an incompatible race of *Phytophthora infestans* of a potato plant cell which has previously been infected by a compatible race. Ann. Phytopathol. Soc. Japan 32: 181–185.
33. Tomiyama, K. 1967. Further observation on the time requirement for hypersensitive cell death of potatoes infected by *Phytophthora infestans* and its relation to metabolic activity. Phytopathol. Z. 58: 367–378.
34. Tyler, A. 1941. Artificial parthenogenesis. Biol. Rev. 16: 291–336.
35. Uehara, E. 1958. On the phytoalexin production of the soybean pod in reaction to *Fusarium* sp., the casual fungus of pod blight. I. Some experiments on the phytoalexin production as affected by host plant conditions and on the nature of the phytoalexin produced. Ann. Phytopathol. Soc. Japan 23: 225–229.
36. Varns, J. L., W. W. Currier, and J. Kuć. 1971. Specificity of rishitin and phytuberin accumulation by potato. Phytopathology 61: 968–971.
37. Wilson, C. L. 1973. A lysosomal concept for plant pathology. Annu. Rev. Phytopathol. 11: 247–272.
38. Zevenhuizen, L. P. T. M. and S. Bartnicki-Garcia. 1969. Chemical structure of the insoluble hyphal wall glucan of *Phytophthora cinnamomi*. Biochemistry 8: 1496–1502.