HOST-PATHOGEN INTERACTIONS IN PLANTS

Plants, When Exposed to Oligosaccharides of Fungal Origin, Defend Themselves by Accumulating Antibiotics

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Plants are exposed to attack by an immense array of microorganisms, yet are resistant to almost all of these potential pests. Many plants respond to an invasion by a pathogenic or nonpathogenic microorganism, whether a fungus, a bacterium or a virus, by accumulating phytoalexins, low molecular weight compounds which inhibit the growth of microorganisms. Phytoalexins are probably also toxic to higher animal and plant cells (38). The production of phytoalexins appears to be a widespread mechanism by which plants attempt to defend themselves against microbes and, perhaps, against other pests (6, 16, 17, 28, 32, 36, 37, 38, 63). Molecules of microbial origin which trigger phytoalexin accumulation in plants have been called elicitors (33). Plants recognize and respond to elicitors as foreign molecules. However, plants are unlikely to have sufficient genetic material to code for unique recognition systems for every bacterial species and strain and every fungal race and virus that plants are exposed to and respond to defensively. Thus, elicitors are likely to be molecules present in many different microbes and, in fact, the elicitor to be described in this paper is a structural polysaccharide of the mycelial walls of many fungi (11).

PHYTOALEXINS – GENERAL ANTIBIOTICS

There are about 100 known phytoalexins distributed among an equally impressive number of plant species. It cannot yet be said, although it is probable, that all plants have the ability to synthesize phytoalexins; but it can be said that the ability to produce phytoalexins is a characteristic of plants that is widespread. Most plants appear to produce several structurally related phytoalexins. Green beans (*Phaseolus vulgaris*) and potatoes (*Solanum tuberosum*) are each capable of accumulating more than half a dozen different but structurally related phytoalexins. The phytoalexins produced by different members of a plant family, although seldom identical, are structurally related. Indeed, the structural relationships between phytoalexins have been used to study the systematic relationships among higher plants (30).

All phytoalexins are lipophilic compounds. The four phytoalexins presented in Fig. 1 are illustrative of the most diverse types of phytoalexins. The most studied phytoalexin of soybeans is glyceollin (Fig. 1) (12). Lyne et al. (42) have characterized two additional soybean phytoalexins which are structural isomers of glyceollin and which appear to have similar antibiotic characteristics. In this manuscript, glyceollin will be a generic term referring to the glyceollin isomers which co-chromatograph on thin-layer chromatography plates. The synthesis of glyceollin, a phenylpropanoid derivative, is probably initiated from phenylalanine via the reaction catalyzed by phenylalanine ammonia-lyase (Fig. 2), but, as yet, no biosynthetic pathway for the production of a phytoalexin has been completely described.

The mechanism by which phytoalexins stop the growth of cells is not understood. Some evidence suggests that phytoalexins alter plasma mem-

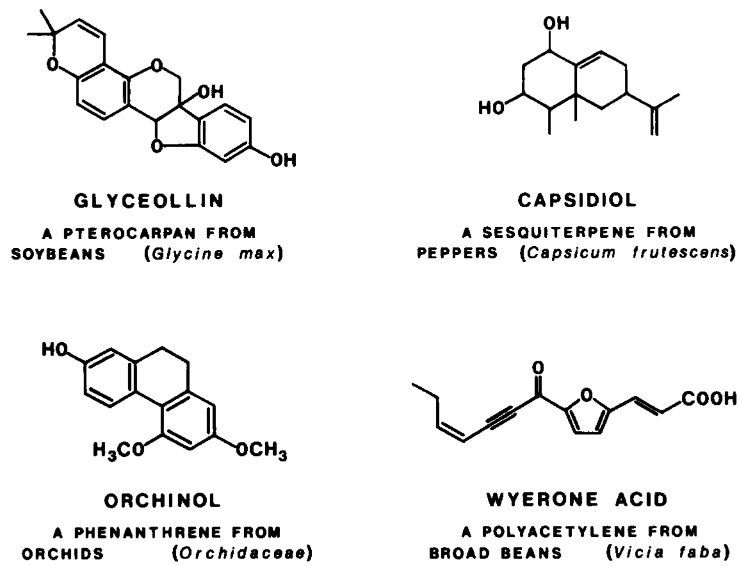

FIGURE 1 Structurally diverse phytoalexins.

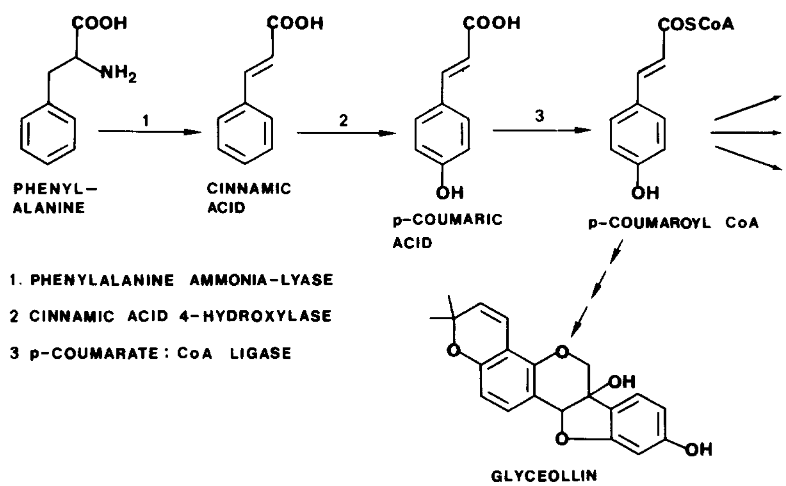

FIGURE 2 Proposed pathway for the biosynthesis of glyceollin, a phytoalexin of soybeans.

branes (46, 52, 62); it has also been suggested that phytoalexins inhibit oxidative phosphorylation (46). On the other hand, some phytoalexins, being furanocoumarins or related compounds (14), are known to cross-link DNA when exposed to ultraviolet light (44). It may be that all or most phytoalexins have a common mode of action. Additional research on the mechanism of action of phytoalexins is needed.

Assaying the degree of toxicity of a phytoalexin can be complicated by the ability of the test organisms to metabolize and thereby detoxify phytoalexins (8, 53, 54). In addition, many phytoalexins appear to be static agents rather than toxic agents. Glyceollin, the soybean phytoalexin, appears to stop the growth of cells without killing them. Glyceollin inhibits the growth, in vitro, of the soybean pathogen *Phytophthora megasperma* var. *sojae* (Pms), the causal agent of root and stem rot. Steven Thomas, in our laboratory, has found that glyceollin will also stop the growth of three Gram-negative bacteria, *Pseudomonas glycinea, Rhizobium trifolii,* and *Rhizobium japonicum*, of the Gram-positive bacterium, *Bacillus subtilis*, and of baker's yeast, *Saccharomyces cerevisiae*. Interestingly, it requires about 25 μg/ml of glyceollin to inhibit by 50% and 100 μg/ml to inhibit by 100% the growth of all of these different organisms (Fig. 3). The fact that it requires the same concentration of glyceollin to inhibit the growth of a variety of cell types suggests that the mechanism of action of glyceollin is a chemically catalyzed reaction (such as the photosensitized furanocoumarins or such as disruption of membranes) rather than an enzyme catalyzed reaction. The results presented in Fig. 3 support the hypothesis that a plant's phytoalexins can potentially protect the plant from a broad spectrum of microorganisms.

Phytoalexins are either not detectable or are detectable in only very low concentrations in healthy plants. Phytoalexins have been frequently referred to as stress metabolites, for cells which are injured or exposed to toxic agents often accumulate phytoalexins. However, challenge of plants by microorganisms uniformly results in the accumulation of large amounts of phytoalexins. Glyceollin is accumulated in large amounts by soybean tissues in response to infection by Pms, the soybean pathogen, as well as in response to challenge by a wide variety of nonpathogenic microorganisms.

ELICITORS OF PHYTOALEXIN ACCUMULATION – ASSAYS

Plants recognize and respond to the presence of microbes by accumulating phytoalexins. Therefore, plants must be recognizing molecules synthesized by these microbes. The first clues to the nature of the molecules which trigger the accumulation of phytoalexins came from studies of pathogens grown in culture. The culture medium, free of the pathogen which had grown in the medium, was shown, in several instances, to elicit phytoalexin accumulation in the pathogen's host (2–4, 22, 23, 33, 35, 48, 49, 59, 64). A desire to know the chemical nature of an elicitor prompted the purification and characterization of the elicitor present in the culture filtrates of Pms, the fungal pathogen which causes root and stem rot in soybeans (5). To purify this biologically active molecule, it was necessary to develop quantitative assays for the biological activity. Arthur Ayers undertook this challenge in the authors' laboratory.

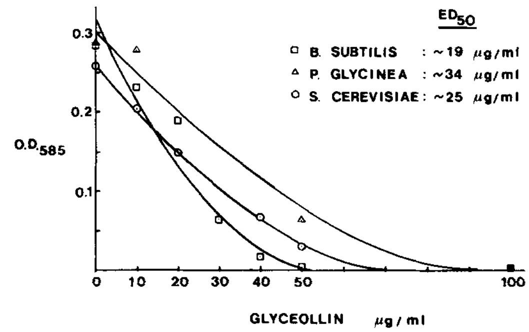

FIGURE 3 Similar concentrations of glyceollin are required to inhibit the growth of *Bacillus subtilis, Pseudomonas glycinia,* and *Saccharomyces cerevisiae*.

Soybean tissues accumulate glyceollin in response to the elicitors present in the fluid of Pms cultures. This response is the basis of three biological assays that were developed. The first assay developed by Ayers was a modification of the cotyledon assay first used by Frank and Paxton (23). For this assay, a section, approximately 1 mm thick and 6 mm is diameter, is cut from the lower surface of cotyledons obtained from 8-day-old soybean seedlings. The sample to be assayed for its ability to elicit the accumulation of the soybean phytoalexin, glyceollin, is placed in a 100-μl drop on the cut surfaces of each of a set of 10 cotyledons. The treated cotyledons are incubated at 26°C on moist filter paper in covered Petri dishes. The wound droplets on the cotyledons turn red (Fig. 4) due to the presence of an unidentified compound called PA_k (23). The red color is usually roughly proportional to the amount of glyceollin in the wound droplet. After 20 h, each set of 10 cotyledons is transferred with forceps to 20 ml of distilled water contained in a 50-ml Erlenmeyer flask. This procedure rinses off the droplets of sample fluid that are retained on the wounded surface. Since glyceollin absorbs light at 285 nm, the absorbance at 285 nm of the wound droplet solution is a measure of elicitor activity. The contribution of glyceollin to the absorbance at 285 nm of wound droplet solutions was determined by isolating the glyceollin from the wound droplet solutions (Fig. 5) (5). The data of Fig. 5 demonstrate that there is a linear relationship between the amount of glyceollin in the wound droplet solutions and the absorbance of the droplets at 285 nm.

FIGURE 4 The cotyledon assay. A section is cut from the lower surface of cotyledons obtained from 8-day-old soybean seedlings. The sample to be assayed for elicitor activity is placed in a droplet on the cut surface. Elicitor causes the colorless phytoalexin, glyceollin, to accumulate in the wound droplets. An unidentified red pigment also accumulates in the wound droplets.

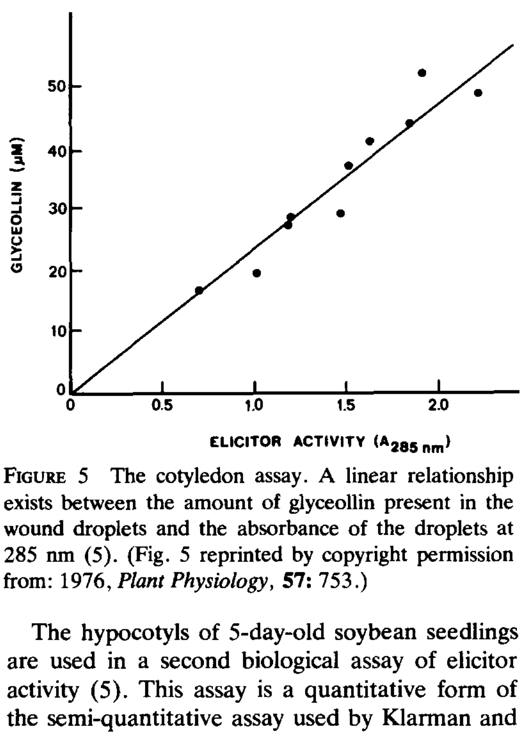

FIGURE 5 The cotyledon assay. A linear relationship exists between the amount of glyceollin present in the wound droplets and the absorbance of the droplets at 285 nm (5). (Fig. 5 reprinted by copyright permission from: 1976, *Plant Physiology*, **57:** 753.)

The hypocotyls of 5-day-old soybean seedlings are used in a second biological assay of elicitor activity (5). This assay is a quantitative form of the semi-quantitative assay used by Klarman and Gerdemann (34) and by Keen (31). The soybean seedlings are removed from the soil and rinsed in tap water. The seedlings are then mounted on horizontal stainless steel needles (1 mm in diameter, 11 cm in length) by piercing the hypocotyls 5 mm below the cotyledons (Fig. 6). The resulting vertical slit wounds in the hypocotyls are about 5 mm in length. A set of 10 seedlings is mounted on each needle, and the seedlings are suspended with their roots in water to within 2 cm of the wounds. A 20-μl drop of an elicitor preparation or a triturated Pms preparation is applied to each hypocotyl wound. Each set of 10 seedlings is removed from its needle after incubation in a closed chamber at 26°C for 24 h at 100% humidity. A 14-mm segment of hypocotyl, centered on the wound, is cut from each seedling, and the glyceollin is extracted and purified from each set of segments. The amount of glyceollin present in spots which are eluted from thin-layer chromatography plates is determined by the absorbance at 285 nm. The data of Fig. 7 demonstrate that, at low elicitor concentrations, there is a linear relationship between the amount of elicitor applied to each hypocotyl and the amount of glyceollin extracted from each hypocotyl (5). It requires $<10^{-11}$ mol of elicitor (~0.2 μg) applied to each

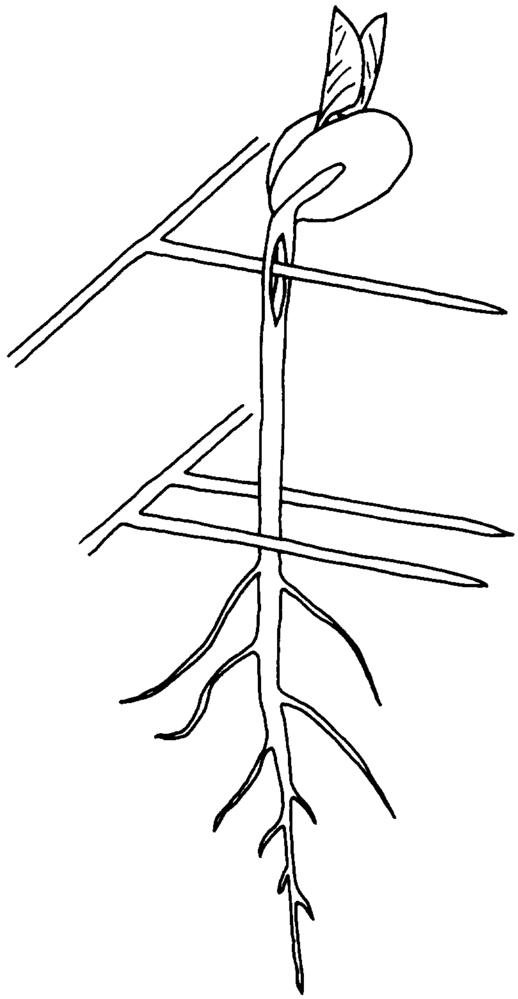

FIGURE 6 The hypocotyl assay. 5-day-old hypocotyl seedlings are mounted on stainless steel needles. Elicitor preparations are placed in the resulting vertical slit wounds. Glyceollin, which accumulates in the tissue surrounding the wound, is extracted, purified, and quantitated.

hypocotyl to result in sufficient glyceollin accumulation to inhibit completely the growth of Pms in vitro.

Elicitors isolated from the culture fluids of three different races of Pms all have equivalent abilities to elicit glyceollin accumulation in the hypocotyls of the soybean cultivar Harosoy 63 (Fig. 7). This observation, and additional data to be described later, demonstrate conclusively that the ability to stimulate phytoalexin production is not in itself sufficient to determine whether a particular race of a pathogen can infect a particular cultivar of its hosts.

Glyceollin is a phenylpropanoid-derived phytoalexin (12). Therefore, elicitation of glyceollin accumulation might also result in the increased activity of the enzymes involved in general phenylpropanoid metabolism. The first such enzyme in the synthesis of phenylpropanoids is phenylalanine ammonia-lyase (Fig. 2). A third assay for elicitors involves the stimulation of the activity of phenylalanine ammonia-lyase and the accumulation of glyceollin in suspension-cultured soybean cells (20). This assay does not depend on wounding of the soybean tissues.

The level of phenylalanine ammonia-lyase increases rapidly (Fig. 8) 3 h after exposure of the suspension-cultured cells to the elicitor (20). The increase in activity of the phenylalanine ammonia-lyase in soybean cell suspension cultures treated with elicitors is followed by the accumulation of glyceollin (Fig. 8). Furthermore, the uptake of nitrate from the medium and the increase in fresh weight of the cells are greatly reduced or stopped in response to the exogenously supplied elicitor (Fig. 9). These results indicate that large changes in the metabolism of cell suspension cultures are induced by the action of the Pms elicitor.

The ability of the Pms elicitor to increase phenylalanine ammonia-lyase activity in suspension-cultured soybean cells is dependent on the amount of elicitor added (Fig. 10). Maximum stimulation of the enzyme activity results from the addition of 1 μg of fraction I Pms elicitor (6, 7). At the time these experiments were carried out, fraction I elicitor was the most purified elicitor preparation available. Elicitor molecules account for only about 10% of the molecules present in fraction IV, and that is why the specific activity of the fraction IV elicitor (Fig. 10) is lower than that of fraction I elicitor. Since the average molecular weight of fraction I elicitor is about 10^5, concentrations as low as 10 nM elicitor result in maximum stimulation of phenylalanine ammonia-lyase activity in these suspension-cultured cells.

The shape of the curves in Fig. 10 deserve further comment. The Pms elicitor is a carbohydrate, as will be described later in this article. The elicitor receptors of plants are likely to be protein – carbohydrate-binding proteins. Carbohydrate-binding proteins are, by definition, lectins. The ability of lectins to bind to carbohydrates on the cell surface of animal cells is well known (39, 40). What is particularly interesting for this discussion about lectins is the shape of the lectin concentration curves obtained when measuring the ability

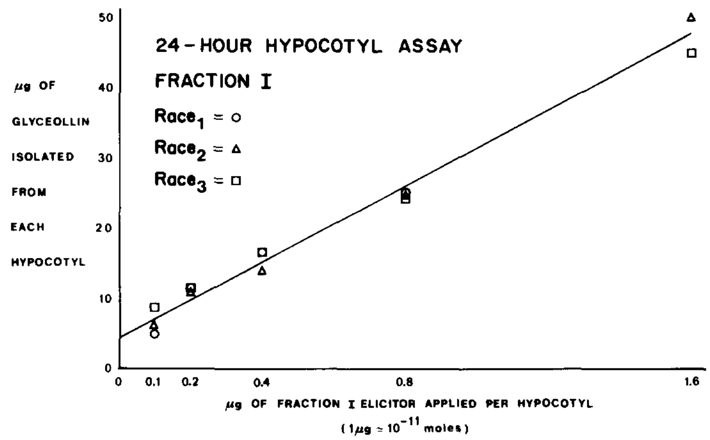

FIGURE 7 The hypocotyl assay. The amount of glyceollin extracted from hypocotyls and purified by thin-layer chromatography is determined by the absorbance at 285 nm. A linear relationship exists, at these low elicitor concentrations, between the amount of elicitor applied and the amount of glyceollin extracted.

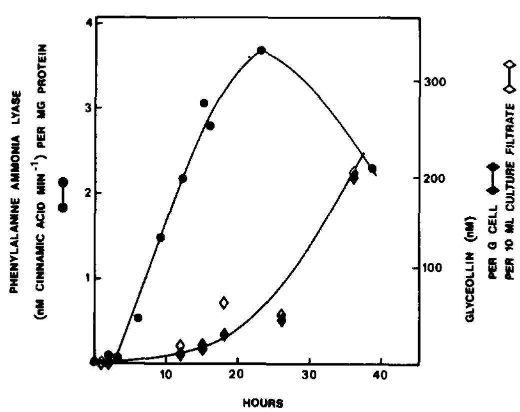

FIGURE 8 Cell-suspension assay. Elicitors stimulate the activity of phenylalanine ammonia-lyase and the accumulation of glyceollin in suspension-cultured soybean cells. Sterile Pms elicitor was added at zero hours (20). (Fig. 8 reprinted by copyright permission from: 1976; *Plant Physiology*, **57**: 777.)

of lectins to bind to animal cell surfaces. The shape of these curves is essentially identical to the shape of the elicitor activity curves illustrated in Fig. 10. A fine example of a lectin-binding curve with this shape is described by Novogrodsky and Ashwell (45) who examined the ability of a lectin, the mammalian hepatic-binding protein, to stimulate mitogenesis in thymus-derived lymphocytes.

Soybean tissues respond to extremely small amounts of Pms elicitor. As little as 10^{-12} mol of an elicitor applied to a single hypocotyl or cotyledon stimulates quantities of glyceollin sufficient to inhibit the growth of Pms and other microorganisms in vitro. It is impressive to observe the effects on the growing suspension-cultured soybean cells caused by the addition of submicromolar quantities of the elicitor. These cells respond to the small amount of elicitor, which is a carbohydrate, even though the cells are growing in the presence of 50 mM sucrose, another carbohydrate. Clearly, the metabolism of the plant cells which are exposed to elicitors is dramatically altered at elicitor concentrations which are equivalent to the concentrations of hormones required for effective metabolic regulation.

THE CHEMICAL NATURE OF THE PMS ELICITOR

The Pms elicitor was first isolated and chemically characterized from old cultures of Pms. The elicitor may have been released into the culture fluid by autolysis (6, 7). It was later demonstrated that elicitor-active molecules with the same chemical and biological properties as the elicitor molecules isolated from Pms culture fluid can be isolated from mycelial walls of Pms (6). The culture fluid and mycelial wall elicitors are heat stable; the elicitor preparations can be autoclaved for 3 h at 121°C without loss of activity. The elicitor molecules are heterogeneous in size, ranging from a mol wt of ~5,000 to ~200,000. The elicitor lacks ionizable groups, as the elicitor does not bind to anion and cation exchange columns under a vari-

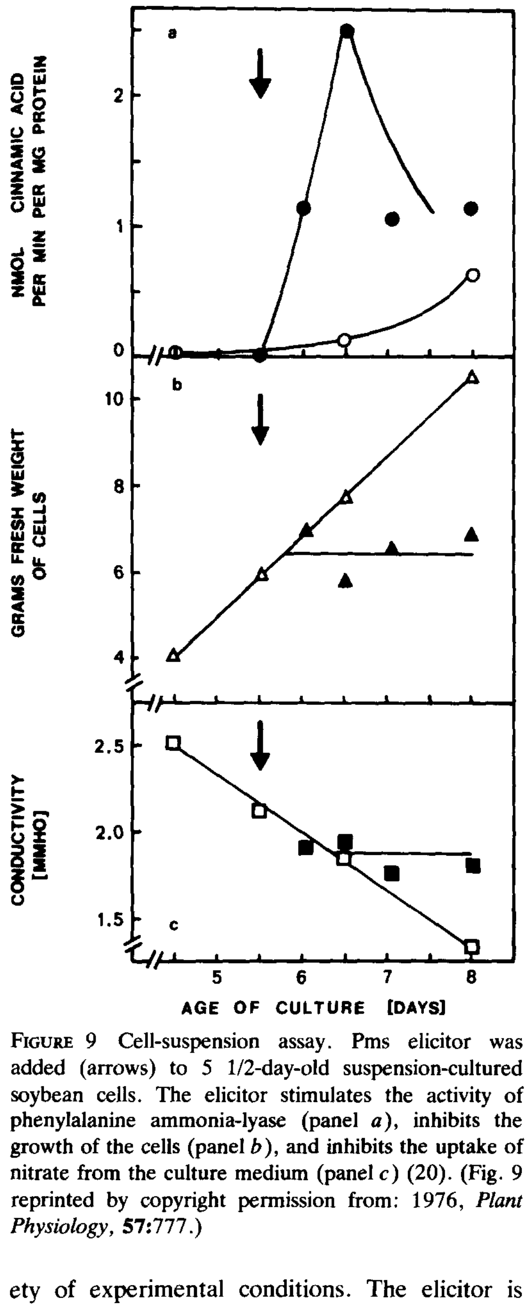

FIGURE 9 Cell-suspension assay. Pms elicitor was added (arrows) to 5 1/2-day-old suspension-cultured soybean cells. The elicitor stimulates the activity of phenylalanine ammonia-lyase (panel *a*), inhibits the growth of the cells (panel *b*), and inhibits the uptake of nitrate from the culture medium (panel *c*) (20). (Fig. 9 reprinted by copyright permission from: 1976, *Plant Physiology*, **57**:777.)

ety of experimental conditions. The elicitor is stable at room temperature between pH 2 and 10. The elicitor is not digested by proteases. All of these properties ruled out the possibility that elicitors are proteins or nucleic acids. On the other hand, these are the expected properties of elicitors, if elicitors are neutral polysaccharides. Indeed, all of the Pms-produced elicitor-active molecules examined have been found to be glucans (5-7, and unpublished results of the authors). The best method for obtaining large amounts of Pms elicitor is partial acid hydrolysis of the mycelial walls (unpublished results of the authors). The series of polysaccharides and oligosaccharides so obtained are extremely active as elicitors of soybean phytoalexins.

Methylation analysis of the Pms elicitors has demonstrated that the elicitors are largely 3-linked polymers with glucosyl branches to C-6 of about one of every three of the backbone glucosyl residues (7, and unpublished results of the authors). Approximately 75% of the glycosidic linkages and 90% of the glycosyl residues in the elicitor-active glucan are hydrolyzed and released by an exo-β-1,3-glucanase isolated from *Euglena gracilis* (10), indicating that the Pms mycelial wall glucan is predominantly a β-linked polymer. Optical rotation and NMR studies have confirmed that the glucan is β-linked (unpublished results of the authors). In fact, this elicitor-active glucan, which constitutes as much as 65% of the Pms mycelial walls, has been demonstrated to be chemically indistinguishable from what is known about the structural β-glucans of the mycelial walls of other *Phytophthora* species (11).

The exo-β-1,3-glucanase isolated from *E. gra-*

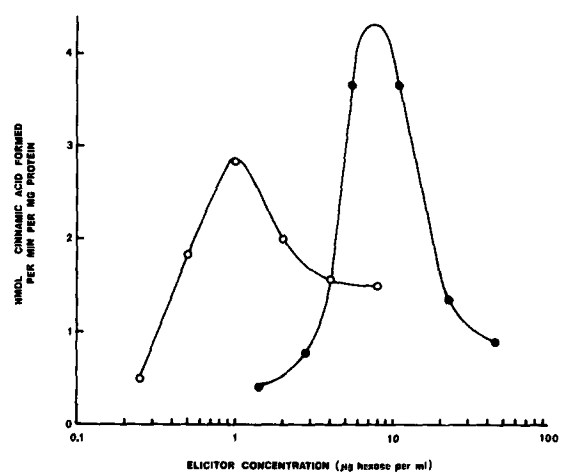

FIGURE 10 Cell-suspension assay. The ability of the Pms elicitor to stimulate phenylalanine ammonia-lyase activity in suspension-cultured soybean cells is dependent on the amount of elicitor added. The open circles represent fraction I Pms elicitor and the closed circles fraction IV Pms elicitor. A more detailed explanation of these fractions is given in the text and in references 6 and 7 (20). (Fig. 10 reprinted by copyright permission from: 1976, *Plant Physiology*, **57**:778.)

cilis has been valuable in the characterization of the Pms elicitor (7, and unpublished results of the authors). That portion of the elicitor, which is released from the walls by aqueous extraction at 121°C, is heterogeneous in size, with an average mol wt of ~100,000. The *E. gracilis* enzyme hydrolyzes glucans from the nonreducing end and is capable of hydrolyzing the glycosidic bond of three-linked glucosyl residues that have other glucosyl residues attached to C-6. The product of the exoglucanase-degraded mycelial wall-released elicitor is still heterogeneous in size, but has an average mol wt of ~10,000 (Fig. 11). This highly branched glucan fragment retains as much activity as the undegraded elicitor. The predominant glycosidic linkages remaining after extensive exoglucanase treatment are 3-linked, 3,6-linked, and terminal glucosyl linkages in a ratio of 1:1:1. Significant amounts of 4-linked and 6-linked glucosyl residues are also present. The fact that the exoglucanase reduces the average size of the elicitor-active molecules by a factor of 10 is evidence that it is the glucan chains that possess the elicitor activity.

The glucan nature of the Pms elicitor has been conclusively established by exposing the elicitor-active oligosaccharides, produced by partial acid hydrolysis of mycelial walls, to the action of a highly purified β-exoglucanase obtained from the cell walls of suspension-cultured soybean cells. This enzyme converts the oligosaccharides to glucose while abolishing the elicitor activity (K. Cline and P. Albersheim, unpublished results).

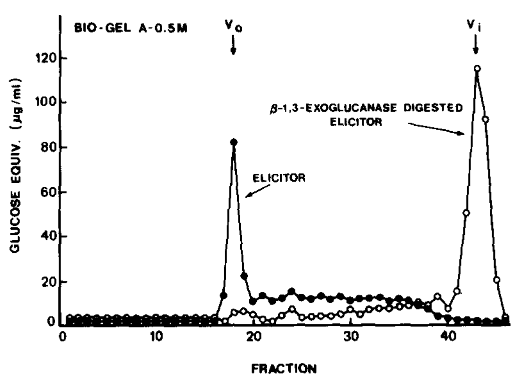

FIGURE 11 A β-1,3-exoglucanase isolated from *Euglena gracilis* reduces by 10-fold the average molecular weight of the mycelial wall-released Pms elicitor. V_0 is the void volume and V_i the included volume of the column (7). (Fig. 11 reprinted by copyright permission from: 1976, *Plant Physiology,* **57**:770.)

Periodate treatment of the wall-released elicitor has confirmed the polysaccharide nature of the active component and suggests an essential role of a branched oligosaccharide having terminal glycosyl residues. Exposing the elicitor to periodate eliminates almost all of the elicitor activity (Fig. 12). On the other hand, a considerable portion of the elicitor activity is regained if the periodate-degraded polymers are reduced with sodium borohydride and then subjected to mild acidic hydrolysis. Since the 3- and 3,6-linked glucosyl residues are resistant to periodate degradation, it seems likely that the periodate has destroyed the elicitor activity by modifying the terminal glucosyl residues or the quantitatively minor but periodate-susceptible 4- and/or 6-linked glucosyl residues. However, recovery of elicitor activity, by mild acid hydrolysis of the periodate-inactivated elicitor, points to periodate attack on the terminal glucosyl residues as the cause for periodate inactivation of the elicitor. The degradation of 4- or 6-linked glucosyl residues would lead to splitting of the glucan chain, while periodate destruction of terminal glucosyl residues followed by mild acid hydrolysis could lead to the exposure of new terminal glucosyl residues which might provide the proper structure of an active elicitor.

The requirement for elicitor activity of a branched oligosaccharide is supported by the observation that 3-linked β-glucans which lack branches to C-6 or have only a single C-6

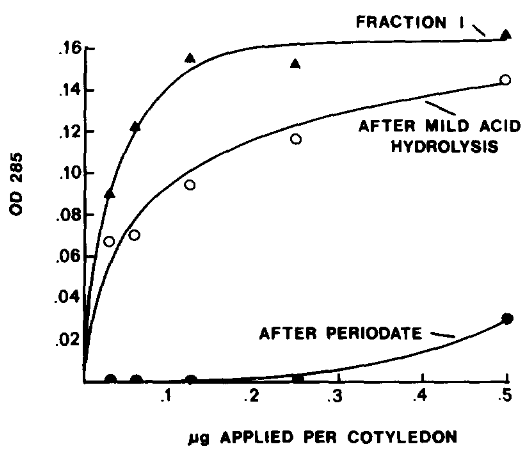

FIGURE 12 Periodate treatment eliminates almost all of the activity (closed circles) of the Pms elicitor (triangles). A considerable portion of the elicitor activity is recovered if the periodate-treated elicitor is reduced with sodium borohydride and then subjected to mild acidic hydrolysis (open circles).

branched glucosyl residue, such as laminarin, have little or no elicitor activity (less than one thousandth of the Pms elicitor). Indeed, a series of commercially available polysaccharides, oligosaccharides, methylglycosides, and simple sugars have been tested for elicitor activity, and, besides laminarin, the only commercially available product found with detectable elicitor activity was nigeran, a mycelial wall glucan from the fungus, *Aspergillus niger* (20).

A major goal of our research is the determination of the detailed molecular structure of the active-site of the Pms elicitor. It is expected that this goal will be achieved by the isolation and structural characterization of the smallest possible elicitor-active oligosaccharide which can be derived from the glucan elicitor. A relatively small elicitor-active oligosaccharide has been produced by partial acid hydrolysis of Pms mycelial walls. The series of oligosaccharides obtained by this partial hydrolysis have been partially resolved, first, by low-resolution (Fig. 13) and then by high-resolution (Fig. 14) Bio-Gel P-2 gel permeation chromatography (Bio-Rad Laboratories, Richmond, Calif.). Oligosaccharides containing as few as nine glucosyl residues still retain elicitor activity. Glucose is the only detected component of these oligosaccharides.

The smallest elicitor-active oligosaccharide-containing fraction from the high resolution P-2 column (Fig. 14) has been subdivided by high pressure liquid chromatography (Fig. 15) into at least five oligosaccharide fractions. Two of the five oligosaccharide fractions obtained by high pres-

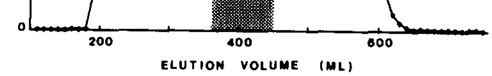

FIGURE 13 Elicitor-active oligosaccharides are produced by partial acid hydrolysis of Pms mycelial walls. The series of oligosaccharides obtained are partially resolved by low-resolution gel permeation chromatography. The smallest elicitor-active oligosaccharides are included in the shaded column fractions.

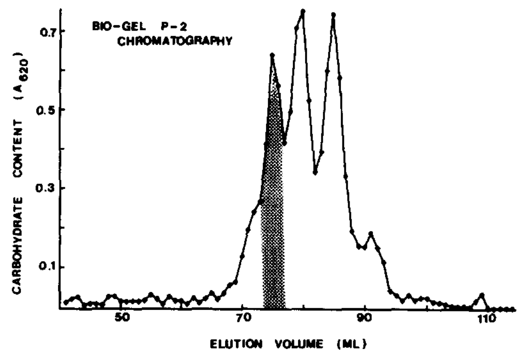

FIGURE 14 The smallest elicitor-active oligosaccharides (shaded fraction illustrated in Fig. 13) are further resolved by high-resolution gel permeation chromatography. The smallest elicitor-active oligosaccharides are included in the shaded column fractions.

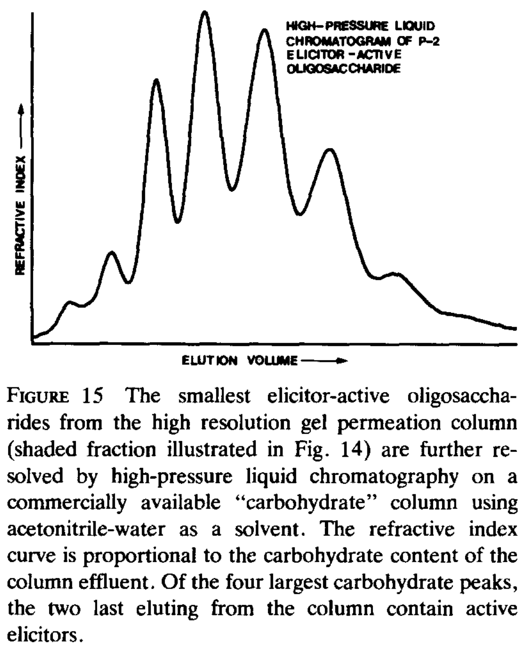

FIGURE 15 The smallest elicitor-active oligosaccharides from the high resolution gel permeation column (shaded fraction illustrated in Fig. 14) are further resolved by high-pressure liquid chromatography on a commercially available "carbohydrate" column using acetonitrile-water as a solvent. The refractive index curve is proportional to the carbohydrate content of the column effluent. Of the four largest carbohydrate peaks, the two last eluting from the column contain active elicitors.

sure liquid chromatography can be degraded by treatment of the mixture of oligosaccharides with the *E. gracilis* exo-β-1,3-glucanase. There appears to be little loss of elicitor activity after treatment with the exoglucanase. Of the three oligosaccharide fractions remaining after exoglucanase treatment, two have elicitor activity. The purest elicitor-active oligosaccharide fraction obtained by high pressure liquid chromatography still contains at least two distinct oligosaccharides. Methylation analysis of this purest elicitor-active oligosaccharide indicates an approximate composition of two 6-linked, two 3-linked, two 3,6-linked, and three terminal glucosyl residues. Indirect evidence suggests to us that the 4-linked glucosyl residues detected in the sample are part of a contaminating inactive oligosaccharide. One possible, very tentative structure of this elicitor-active oligosaccharide is illustrated in Fig. 16 (unpublished results of the authors). Further purification is required before a definitive structure of an elicitor-active oligosaccharide can be obtained.

ELICITORS LACK RACE SPECIFICITY

The three Pms races (races 1, 2, and 3) are distinguished by their differing abilities to infect various soybean cultivars. However, the elicitors of phytoalexin accumulation are not the specificity determining factors in the Pms-soybean system. The elicitor obtained from each of the three Pms races purifies in exactly the same manner, and all the discernible structural features of the elicitors from the three races are identical (7). The activities of the elicitors purified from the three Pms races were carefully examined using the three separate bioassays: the cotyledon assay (Fig. 17) (5), the hypocotyl assay (Fig. 18) (5), and the cell suspension-culture assay (20). All three assays give the same results, that is, the activities of the elicitors from different Pms races are identical (7). This is true for low levels of applied elicitor as well as for levels of elicitor which stimulate maximum glyceollin accumulation (Figs. 7, 17, and 18). Therefore, the three races of Pms are equally

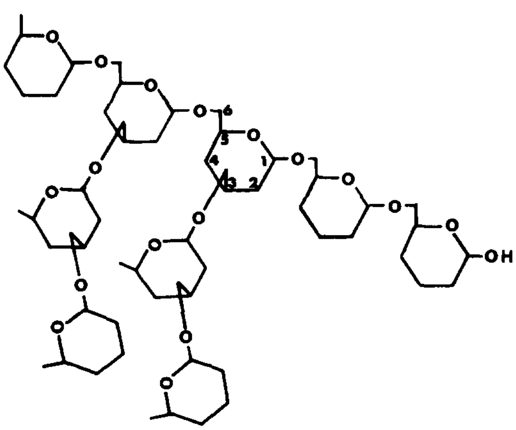

FIGURE 16 One possible, very tentative structure of an elicitor-active oligosaccharide is illustrated. Further purification of the elicitor-active oligosaccharides is required before a definitive structure can be obtained.

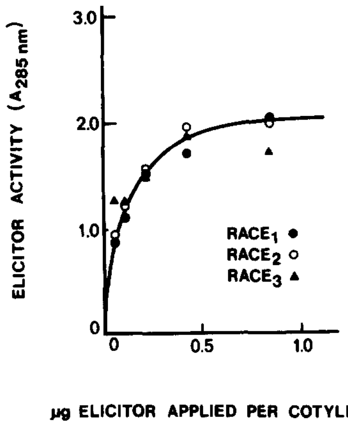

FIGURE 17 The elicitors purified from three different Pms races have an identical ability to stimulate glyceollin accumulation in soybean cotyledons.

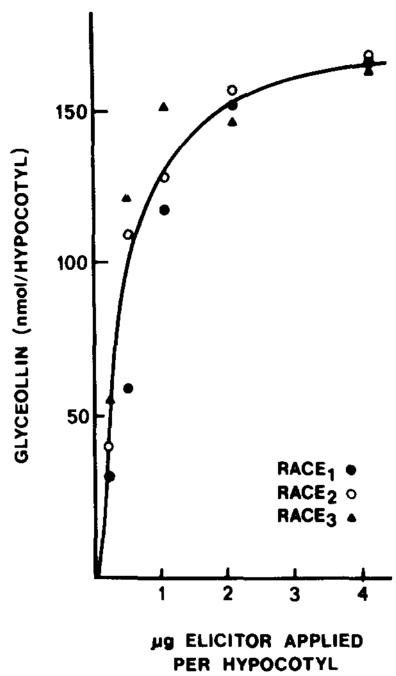

FIGURE 18 The elicitors purified from three different Pms races have an identical ability to stimulate glyceollin accumulation in soybean hypocotyls.

effective at stimulating phytoalexin accumulation in the tissues of susceptible and resistant cultivars of their host. The results contradict the earlier report of Keen (31) which claimed that the Pms elicitors are race specific.

The results of another type of experiment support our conclusion that elicitors are not responsible for race-specific resistance in the Pms-soybean system. Soybean hypocotyls accumulate glyceollin when inoculated with living mycelia of Pms. The response, which is characteristic of natural infections with either an infective or a noninfective race of Pms, is retained with this inoculation technique. We have compared the relative effectiveness of live mycelia and purified elicitor in stimulating glyceollin accumulation (6). The result is the following: The onset and the rate of glyceollin accumulation in seedlings inoculated with infective (compatible) mycelia is indistinguishable from the onset and rate of glyceollin accumulation in seedlings inoculated with either noninfective (incompatible) mycelia or purified elicitor (Fig. 19). These results demonstrate that differences in the rates of glyceollin accumulation in response to different races of Pms are not likely to account for the resistance or susceptibility of various soybean cultivars to the Pms races.

The available evidence does indicate that elicitors have a role in resistance even though they are

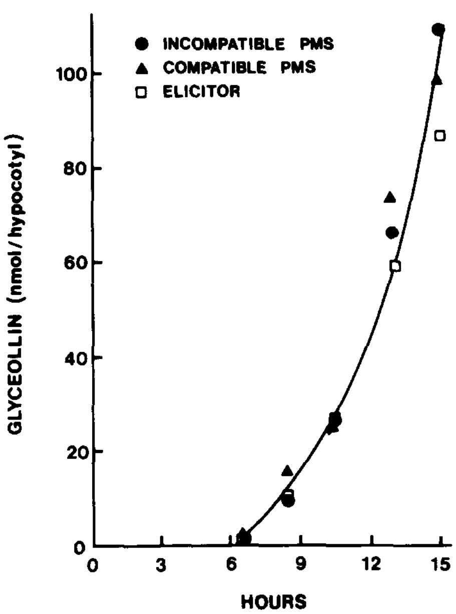

FIGURE 19 The onset and the rate of glyceollin accumulation in the hypocotyls of soybean seedlings inoculated with the mycelia of an infective (compatible) race of Pms is indistinguishable from the onset and rate of glyceollin accumulation in seedlings inoculated either with a noninfective (incompatible) race of Pms or with purified Pms elicitor (6). (Fig. 19 reprinted by copyright permission from: 1976, *Plant Physiology*, **57**:764.)

not determinants of race specificity. The elicitor isolated from Pms is capable of protecting soybean hypocotyls from infection by a normally infective race of Pms if the elicitor is applied to the hypocotyls 6 h before inoculation with Pms (6, and unpublished results of the authors). The elicitor cannot protect soybean tissue when applied simultaneously with an infective race of Pms (Fig. 19).

ELICITORS ARE WIDESPREAD IN NATURE

Soybean plants have evolved the ability to recognize and respond to the structural β-glucan of *Phytophthora* mycelial walls. Similar β-glucans are found in the walls of a wide range of fungi (11). One fungus containing such β-glucans is brewer's yeast, *S. cerevisiae*, a nonpathogen of plants. An elicitor has now been purified from a commercially available extract of brewer's yeast (Difco Laboratories, Detroit, Mich.) (25). The 80% ethanol insoluble fraction of the yeast extract contains a very active elicitor of glyceollin accumulation in soybeans. Most of the polysaccharide in this 80% ethanol insoluble fraction is mannan. However, yeast extract does contain small amounts of the β-glucan. The glucan can be almost completely separated from the mannan by binding the mannan to an affinity column consisting of concanavalin A covalently attached to Sepharose. The glucan can be separated from glycoproteins by binding the proteins to sulfopropyl-Sephadex. Both the purified mannan and purified glucan remain contaminated by small amounts ($\simeq$2%) of arabinogalactan. Ribose-containing polymers, which contaminate the 80% ethanol insoluble fraction, are removed on a diethylaminoethyl-cellulose column. The elicitor activity of the crude 80% ethanol precipitate of yeast extract resides in the glucan component (Fig. 20). The small amount of residual activity remaining in the mannan fraction can be attributed to the observed contamination of this fraction by the glucan (25). The glucan is composed of the same glucosyl linkages found in the Pms elicitor (Fig. 21).

The same quantities of the yeast and Pms elicitors are required for 50% of maximum stimulation of glyceollin accumulation in the cotyledons (Fig. 22 A) and hypocotyls (Fig. 22 B) of soybean seedlings (Fig. 22). Although the yeast elicitor fails to stimulate the same maximum level of glyceollin accumulation in the soybean tissues as

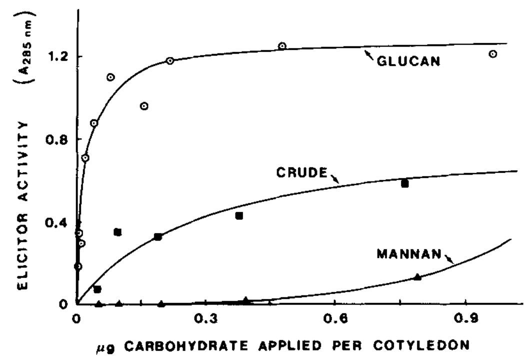

FIGURE 20 Yeast extract contains a very active elicitor of glyceollin accumulation in soybeans. The elicitor activity present in the crude 80% ethanol precipitate of yeast extract (solid squares) residues in a glucan (open circles). The small amount of elicitor activity remaining in the mannan-containing fraction (closed triangles) can be attributed to contamination of this fraction by the glucan. (Fig. 20 reprinted by copyright permission from: 1978, *Plant Physiology*.)

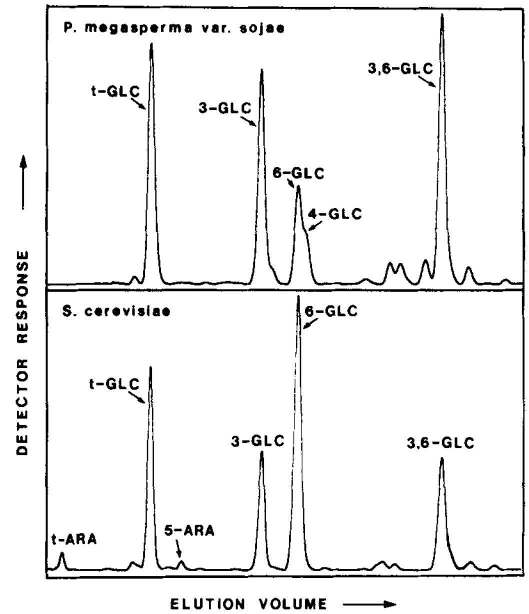

FIGURE 21 The yeast (*Saccharomyces cerevisiae*) glucan is composed of the same glucosyl linkages found in the Pms (*Phytophthora megasperma* var. *sojae*) elicitor. This figure compares the gas chromatographically separated, partially methylated alditol acetates derived from the elicitors. The partially methylated alditol acetates were detected by flame ionization. Examples of the nomenclature are: *t-GLC* represents a glucosyl residue glycosidically linked to another glucosyl residue through C-1. *3-GLC* represents a glucosyl residue glycosidically linked to another glucosyl residue through C-1 and which has a second glucosyl residue glycosidically linked to it at C-3. The terminal arabinosyl (*t-ARA*) and 5-linked arabinosyl (*5-ARA*) residues are thought to be part of a contaminating polysaccharide (25). (Fig. 21 reprinted by copyright permission from: 1978, *Plant Physiology*.)

is maximally stimulated by the Pms elicitor, especially in hypocotyls (Fig. 22B), the amount of glyceollin that is accumulated is more than sufficient to stop the growth of yeast and Pms in culture. The reason for the differential maximum stimulation of glyceollin accumulation is not known, although it could result from a more rapid degradation of the yeast elicitor by glucanases present in the soybean tissue (K. Cline and P. Albersheim, unpublished results).

Our laboratory has obtained other evidence that elicitors have the ability to stimulate phytoalexin accumulation in a wide variety of plants. For example, the Pms elicitor stimulates suspension-cultured cells of sycamore and parsley to produce large amounts of phenylalanine ammonia-lyase activity (20). In addition, we have obtained evidence that the Pms elicitor stimulates *P. vulgaris,* the true bean, to accumulate its phytoalexins (K. Cline and P. Albersheim, unpublished results). This is demonstrated by a different type of bioassay test for phytoalexins. In this bioassay, extracts of plants which may contain phytoalexins are applied to a thin-layer chromatography plate. The phytoalexins are then separated by chromatography in organic solvents. After development of the plate, the organic solvents are allowed to evaporate. The plates are then sprayed with nutrient agar and with the black spores of the fungus *Cladosporium cucumerinum*. The fungus grows on the nutrient agar except in areas where the presence of phytoalexins inhibits the growth of the fungus. These areas remain white on a dark background due to the pigmented spores of the fungus. The *C. cucumerinum* bioassay (Fig. 23) demonstrates that the Pms elicitor stimulates the cotyledons of true beans to accumulate several phytoalexins (K. Cline and P. Albersheim, unpublished results). The same bioassay (Fig. 23) illustrates that both the spores and a mycelial wall extract of *Colletotrichum lindemuthianum* elicit phytoalexin accumulation in the true bean cotyledons. *Colletotrichum lindemuthianum* is a fungal pathogen of true beans. Evidence has been presented that the elicitor present in the mycelial walls of *C. lindemuthianum* is a glucan, but quite

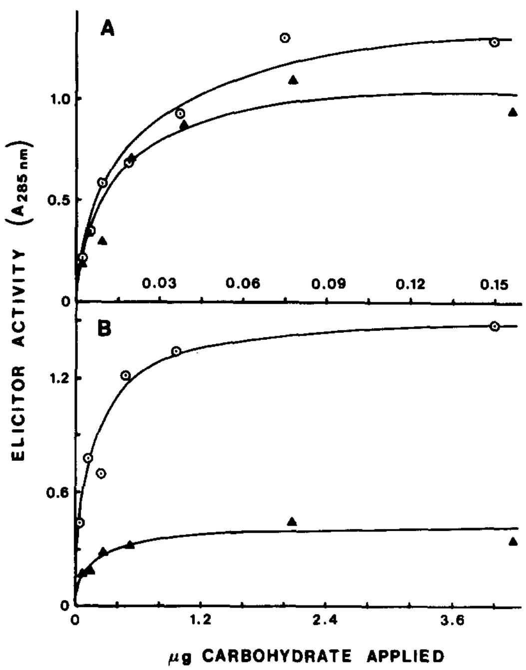

FIGURE 22 The same quantities of the yeast (triangles) and of the Pms (circles) elicitors are required for 50% of maximum stimulation of glyceollin accumulation in the cotyledons (A) and the hypocotyls (B) of soybean seedlings. In every case, the amount of glyceollin accumulated is more than sufficient to stop the growth of yeast and of Pms in culture (25). (Fig. 22 reprinted by copyright permission from: 1978, *Plant Physiology*.)

a different glucan from the Pms and yeast elicitors (3).

Other experiments in our laboratory (M. Wade and P. Albersheim, unpublished results) demonstrate conclusively that elicitors are widespread in nature and that diverse plants are able to respond to a single elicitor. In these experiments, both the Pms and yeast elicitors stimulate potatoes to accumulate rishitin (Fig. 24), a phytoalexin found in potatoes. The levels of rishitin accumulated are the same as the levels accumulated when potatoes are challenged by pathogenic and nonpathogenic fungi (15, 50, 51, 58).

The generality of the elicitor concept has been supported by Lisker and Kuć (41) who have found that diverse fungi, including several *Phytophthora* spp., stimulate potatoes to accumulate several phytoalexins. Indeed, Lisker an Kuć found that glucans isolated from three of the *Phytophthora* spp. also elicited the accumulation of the phytoalexins.

PHYTOALEXINS ARE NOT CAPABLE, BY THEMSELVES, OF PROTECTING PLANTS FROM THEIR PATHOGENS

A microorganism which has evolved the ability to grow successfully on a plant and thus become pathogenic to that plant must also have evolved a mechanism of avoiding the toxic effects of phytoalexins. There are a number of plausible mechanisms for such avoidance by successful pathogens. One such mechanism might be simply the ability of an infective strain of a pathogen to grow away from the areas in which the plant is accumulating toxic levels of phytoalexin. This possibility may be an explanation for the avoidance of the effects of glyceollin in soybean by infective races of Pms.

There are other mechanisms by which a pathogen might prevent a plant from stopping the growth of the pathogen by accumulation of phytoalexins. For example, a pathogen might secrete a toxin which kills the plant cells in the region of the pathogen before those cells are capable of synthesizing the enzymes necessary for synthesis of phytoalexins. Pathogens are well known for their ability to secrete phytotoxins (24, 47, 55–57). Another possible mechanism by which a successful pathogen might prevent a plant from accumulating sufficient phytoalexins might be to repress synthesis of one or more enzymes involved in phytoalexin synthesis or else to inhibit the enzymes once they are synthesized. This possibility has not been examined yet because the biosynthetic pathways of phytoalexins are unknown. A known mechanism by which some pathogens overcome phytoalexin inhibition is the enzymatic conversion of the phytoalexins to less toxic or unstable compounds (13, 18, 19, 21, 26, 27, 29, 43, 60, 61, 63).

Pathogens might mask their presence in their host and thus avoid eliciting phytoalexin accumulation. Pathogens could accomplish this by secreting proteins or other molecules which specifically inhibit the enzymes of the host which solubilize elicitors from the mycelial walls of the pathogen. Evidence suggestive of this type of mechanism has been obtained (1). The existence of host-enzymes capable of solubilizing elicitors has been established (K. Cline and P. Albersheim, unpublished results). Finally, pathogens might mask their presence by secreting a carbohydrate which effectively but innocuously competes with the elicitor for binding to the elicitor's receptor.

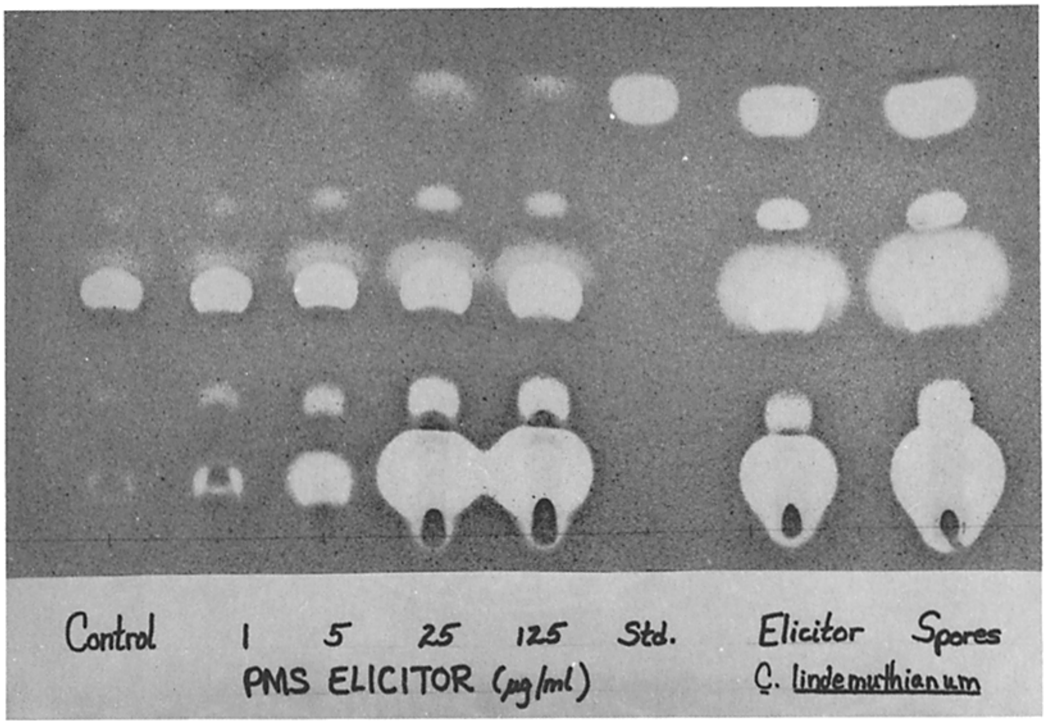

FIGURE 23 A bioassay for phytoalexins. Phytoalexins (white spots) inhibit the growth of the dark spores of *Cladosporium cucumerinum* in nutrient agar which has been sprayed on a thin-layer chromatography plate containing the phytoalexins. The phytoalexins were extracted from the cotyledons of true beans (*Phaseolus vulgaris*) that had been exposed to elicitors or to spores of *Colletotrichum lindemuthianum*. The *C. lindemuthianum* spores germinate and the resulting mycelia grow into and elicit the accumulation of phytoalexins in the cotyledons.

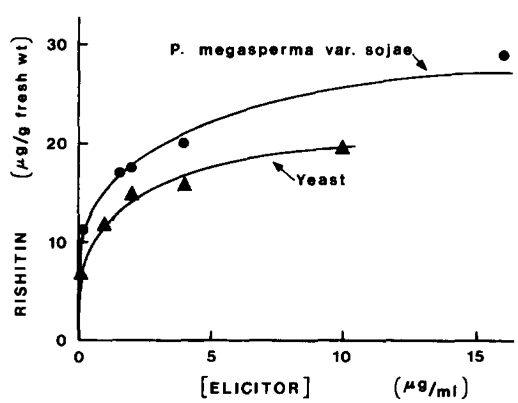

FIGURE 24 The Pms and yeast elicitors both stimulate potato tubers to accumulate the phytoalexin rishitin.

CONCLUDING REMARKS

The ability of plants to synthesize phytoalexins, molecules which have an ability to stop the growth of a wide variety of cells, is one mechanism by which plants attempt to defend themselves. This mechanism appears to be a widespread and effective way for plants to defend themselves against those microorganisms which have not become pathogenic. In other words, the ability to synthesize phytoalexins is a mechanism by which plants are able to stop the growth of microorganisms which have not become pathogenic on the phytoalexin-producing plant. Although not sufficient for resistance to its pathogens, an ability to synthesize phytoalexins is likely to be essential for a plant to be resistant to pathogens.

Plants must recognize that nonpathogenic microorganisms are present in order for plants to activate phytoalexin synthesis and thereby defend themselves against nonpathogenic microorganisms. Plants recognize the presence of many nonpathogenic fungi by recognizing a structural component of the mycelial walls of the fungi. In doing so, plants have evolved a mechanism for recognizing a component of fungi which is essential for the survival of the fungi. Therefore, fungi cannot readily evade detection by altering the structure of this component. Other microorganisms do not have structural glucans in their walls which are similar to the Pms and yeast glucans. Bacteria, for example, are not known to contain these glucans. There is likely to be some other component of bacteria, or perhaps several components, which act as elicitors in plants. It is well known that bacteria do elicit phytoalexin production in plants. We are now attempting to identify a bacterial elicitor. Plants are also known to respond to virus infection by producing phytoalexins (9). Little is known about whether plants respond to the presence of phytophagous insects by synthesizing phytoalexins (32).

The ability of an oligosaccharide to act as a regulatory molecule is of general biological interest. The Pms elicitor is an oligosaccharide composed only of glucose. This oligosaccharide can dramatically alter the metabolism of receptive cells. Thus, oligosaccharides must be added to proteins, glycoproteins, peptides, and steroids as molecules which can act as regulatory molecules.

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