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Plants Interact With Microbial Polysaccharides

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Plants are resistant to almost all of the microorganisms with which they come in contact. In response to invasion by a fungus, bacterium, or a virus, many plants produce low molecular weight compounds, phytoalexins, which inhibit the growth of microorganisms. Phytoalexins are produced whether or not the invading microorganism is a pathogen. The production of phytoalexins appears to be a widespread mechanism by which plants attempt to defend themselves against pests. Molecules of microbial origin which trigger phytoalexin accumulation in plants are called elicitors. Structural polysaccharides from the mycelial walls of several fungi elicit phytoalexin accumlation in plants. Approximately 10 ng of the polysaccharide elicits the accumulation in plants of more than sufficient amounts of phytoalexin to stop the growth of microorganisms in vitro. The best characterized elicitors have been demonstrated to be β-1,3-glucans with branches to the 6 position of some of the glucosyl residues. Oligosaccharides, produced by partial acid hydrolysis of the mycelial wall glucans, are exceptionally active elicitors. The smallest oligosaccharide which is still an effective elicitor is composed of about 8 sugar residues.

Bacteria also elicit phytoalexin accumulation in plants, but the Rhizobium symbionts of legumes presumably have a mechanism which allows them to avoid either eliciting phytoalexin accumulation or the effects of the phytoalexins if they are accumulated. The lectins of legumes bind to the lipopolysaccharides of their symbiont, but not of their non-symbiont, Rhizobium. It is not known whether the lectin-lipopolysaccharide interaction is involved with the establishment of symbiosis. However, evidence will be presented that suggests that lectins are, in fact, enzymes capable of modifying the structures of the lipopolysaccharides of their symbiont, but not of their non-symbiont, Rhizobium. It will also be shown that the lipopolysaccharides isolated from different Rhizobium species and from different strains of individual Rhizobium species have different sugar compositions. Thus, the different strains of a single Rhizobium species are as different from one another as the different species of Salmonella and other gram-negative bacteria. This conclusion is substantiated by experiments demonstrating that antibodies to the lipopolysaccharide from a single Rhizobium strain can differentiate that strain from other strains of the same species as well as from other Rhizobium species. The role in symbiosis of the strain-specific O-antigens is unknown.

Key words: plants, polysaccharides, elicitors, phytoalexins, Rhizobium, nitrogen-fixation

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I. ELICITORS OF PHYTOALEXIN ACCUMULATION IN PLANTS

A. Introduction

Plants are exposed to attack by an immense array of microorganisms, and yet plants are resistant to almost all of these potential pests. A microorganism which is a pathogen of a plant is a special case; pathogens have developed the ability to overcome the plant's defenses. A microorganism which can form a symbiotic relationship with a plant is also a rare case, and again the symbiont must overcome the plant's defense mechanisms. Our research group has been trying to understand how plants resist infection by the vast majority of microorganisms with which they come in contact.

B. Phytoalexins and Elicitors

Plants do not have an immune system similar to that of animals. Instead of antibodies, etc., plants produce low-molecular-weight compounds, phytoalexins, which inhibit the growth of microorganisms. Phytoalexins are produced in response to invasion by a fungus, a bacterium, or a virus. The production of phytoalexins appears to be a widespread mechanism by which plants attempt to defend themselves against pests (1--3). The molecules of microbial origin which trigger phytoalexin accumulation in plants have been called elicitors (4). Plants recognize and respond to elicitors as foreign molecules. It is highly improbable that plants have evolved separate recognition systems for every bacterial species and strain and every fungal race and every virus that plants are exposed to. Thus, elicitors are likely to be molecules common to many microbes and, in fact, the only elicitor to be well characterized and the one to be described in this paper is a fungal polysaccharide, a polysaccharide which is a structural component of the mycelial walls of many fungi.

Most plants produce several structurally related phytoalexins. The most studied phytoalexin of soybeans is glyceollin (5). Lyne et al. (6) have characterized 2 additional soybean phytoalexins which are structural isomers of glyceollin and which appear to have similar antibiotic characteristics. Glyceollin is a phenylpropanoid derivative, and thus its synthesis is probably initiated from phenylalanine via the reaction catalyzed by phenylalanine ammonia lyase (Fig. 1).

Steven Thomas in our laboratory has been studying the effect of glyceollin on a variety of microorganisms. Glyceollin is a static agent rather than a toxic agent, a trait which seems to be common to many, if not all, phytoalexins. Thomas has found that glyceollin will stop the growth of a Gram-negative bacterium, Pseudomonas glycinea, a Gram-positive bacterium, Bacillus subtilis, and baker's yeast, Saccharomyces cerevisiae. Interestingly, it requires about 25 μg/ml of glyceollin to inhibit by 50% the growth of all three of these different organisms (Fig. 2). Thus, it appears that a plant's phytoalexins can potentially protect the plant from a broad spectrum of microorganisms.

C. Assays of Elicitor Activity

Resistance of soybean (Glycine max var. L.) seedlings to Phytophthora megasperma var. sojae (Pms), the causal agent of root and stem rot, is in part due to the accumulation of glyceollin at the site of infection (7). The accumulation of glyceollin is triggered by infection but also by elicitors which are polysaccharides purified from Pms. The ability of Pms elicitor to stimulate glyceollin accumulation in soybean tissues was used as the basis for biological assays of elicitor activity. Bioassays were developed and characterized using the cotyledons (seed leaves) and the hypocotyls (upper stems) of soybean seedlings (8). A

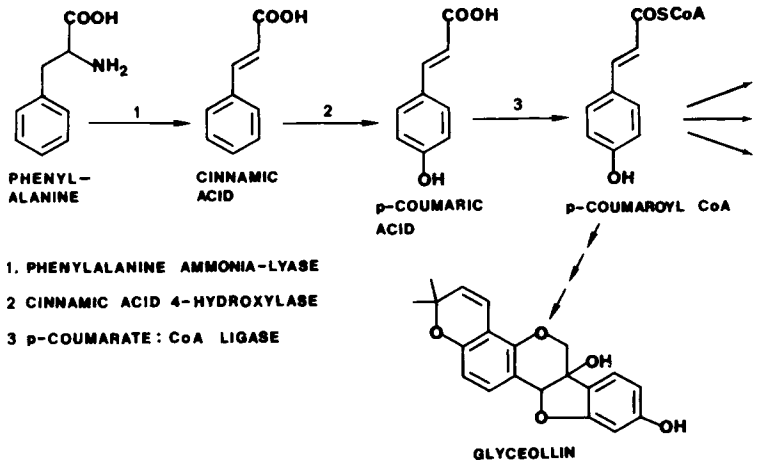

Fig. 1. Presumed biosynthetic pathway of glyceollin. Glyceollin is a phytoalexin produced by soybeans. Phytoalexins are capable of stopping the growth of microorganisms.

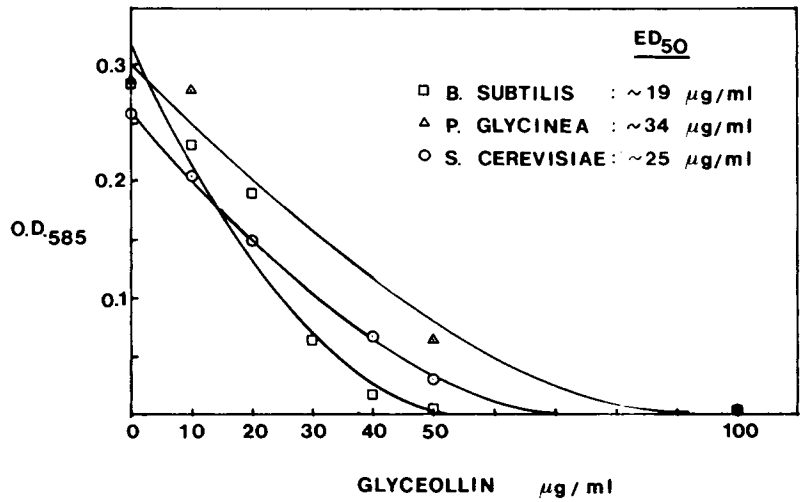

Fig. 2. The effect of glyceollin on the growth [optical density (O.D.) at 258 nm] of Bacillus subtilis, Pseudomonas glycinea, and Saccharomyces cerevisiae.

third bioassay was developed using suspension-cultured cells of soybeans (9). In all 3 assays, the production of glyceollin in plants is proportional to the amount of elicitor applied. This is illustrated for the hypocotyl assay in Fig. 3. In this assay, the glyceollin is

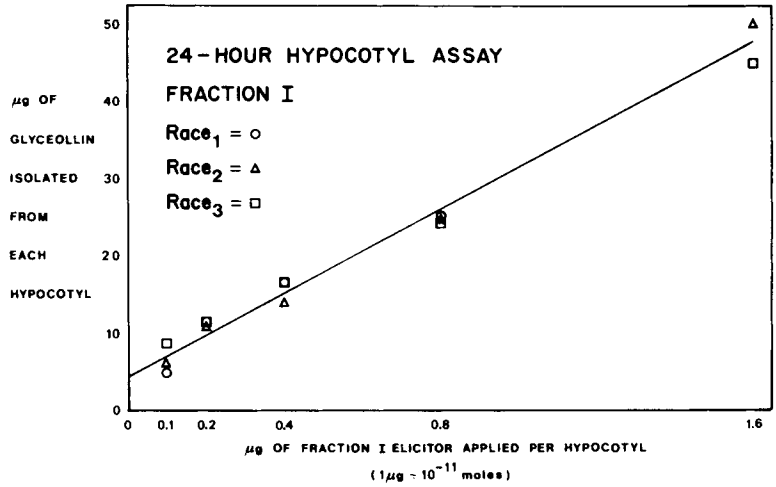

Fig. 3. The elicitors isolated from Phytophthora megasperma var. sojae races 1, 2, and 3 have equal abilities to stimulate glyceollin accumulation in the hypocotyls of 5-day-old soybean seedlings (Glycine max L. cu. Harosoy 63). Harosoy 63 is incompatible with (resistant to) Pms races 1 and 2 and compatible with (susceptible to) Pms race 3.

extracted from the hypocotyls by ethanol and then separated from contaminating compounds by thin layer chromatography. The amount of the ultraviolet absorbing glyceollin on the thin layer plates is quantitated by scraping the glyceollin from the silica gel, and measuring its absorbance at 285 nm. The identity of the glyceollin was confirmed by co-chromatography with standard glyceollin in several solvents on thin layer plates as well as by combined gas chromatography and mass spectrometry of the acetate derivative.

The cotyledon assay was used for purification of the elicitor as the cotyledon assay is less laborious than the hypocotyl assay. The cotyledon assay is based on the fact that when water droplets containing elicitor are placed on the cut surface of cotyledons, some of the glyceollin that is synthesized diffuses into the water droplets. These droplets are diluted and their absorbance at 285 nm is measured. The absorbance of this solution is reasonably proportional to the amount of glyceollin in the solution (8) even though other 285 nm absorbing compounds are also present.

D. The Effect of Pms Elicitor on Soybean Tissues

Elicitors, when introduced into flasks containing suspension-cultured soybean cells, have a dramatic effect on the cells (9). Within a few hours, the cells turn light brown. At the same time, the activity in the cells of at least one of the enzymes involved in the synthesis of glyceollin, phenylalanine ammonia lyase, is greatly increased (Figs. 1 and 4). The increase in activity of the phenylalanine ammonia lyase precedes the accumulation of glyceollin both in the cells and in the culture medium (Fig. 4). The growth of the suspension-cultured cells, as measured by fresh weight, stops upon addition of the elicitor (Fig. 5b). The cells also stop taking up ions from the media, which is another indication of the lack of growth by these cells (Fig. 5c).

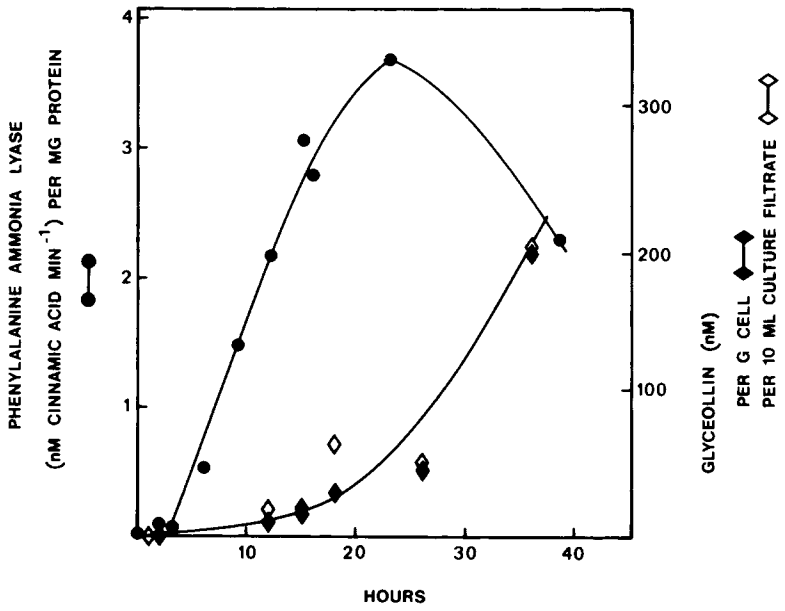

Fig. 4. The addition of 1 μg/ml of Pms elicitor to suspension-cultured soybean cells results in an increase, after about 5 hr, in the activity of phenylalanine ammonia lyase and the accumulation, after about 10 hr, of glyceollin in both the cells and in the culture fluid. Phenylalanine ammonia lyase is thought to be involved in the synthesis of glyceollin (Fig. 1).

Soybean tissues are sensitive to extremely small amounts of Pms elicitor. It is impressive to observe the effects on the growing suspension-cultured soybean cells caused by the addition of submicromolar quantities of the polysaccharide elicitor even though the cells are growing in the presence of 50 mM sucrose. The data of Fig. 3 illustrates the sensitivity of the soybean hypocotyls to the presence of the elicitor. About 10^{-12} moles of elicitor applied to a single hypocotyl stimulates quantities of glyceollin sufficient to prevent the growth of Pms and other microorganisms in vitro.

E. The Chemical Nature of the Pms Elicitor

The evidence that demonstrated that the elicitor is a polysaccharide included the fact that the elicitor is stable to autoclaving at 121°C for several hours, lacks affinity for both anion and cation exchange resins, is completely stable to treatment by pronase, and in size heterogeneous. The elicitor was first found in the fluid of old cultures of Pms and was probably released into the culture fluid by autolysis of some of the mycelia. It was later demonstrated that elicitor-active molecules with the same properties as the culture fluid elicitor could be isolated from the mycelial walls of Pms by a heat treatment similar to that used to solubilize the surface antigens from the cell walls of S. cerevisiae (10). It has now been demonstrated that the elicitor-active wall-released molecules are β-glucans. Methylation analysis of the purified elicitor has demonstrated that this glucan is largely a 3-linked polymer with glucosyl branches to about 1 out of every 3 of the backbone glucosyl residues (Table I, column 1).

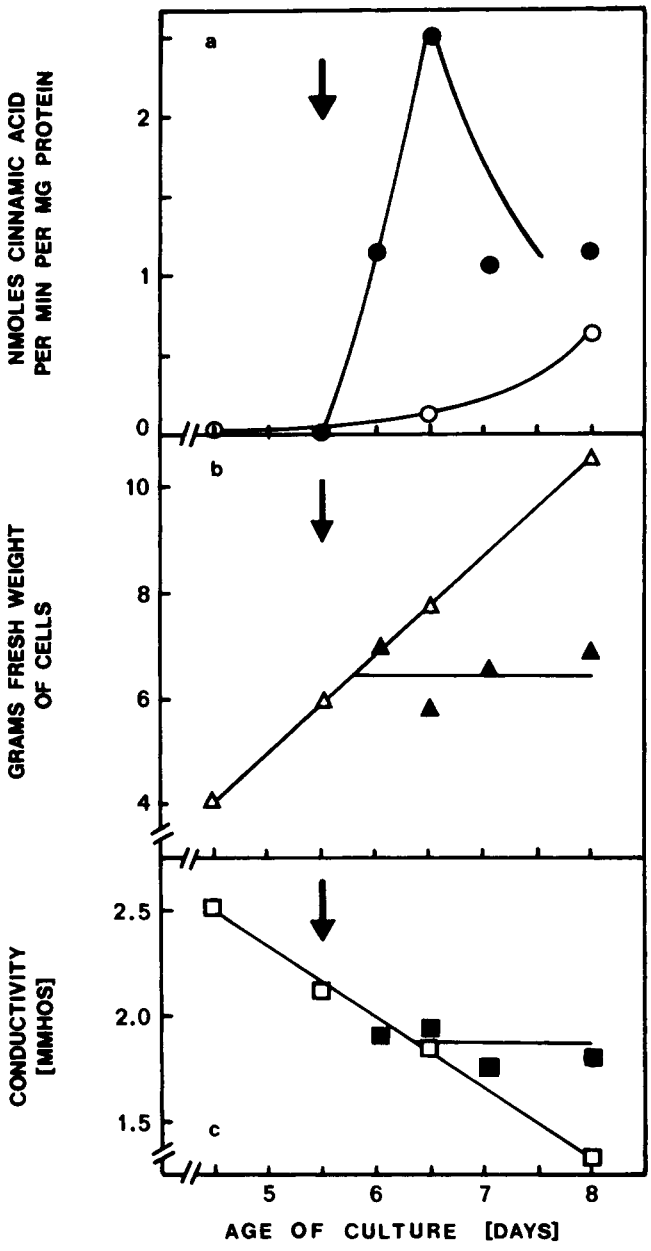

Fig. 5. The activity of phenylalanine ammonia lyase (a) increases following the addition (arrow) of 1 μg/ml of Pms elicitor to log phase suspension-cultured soybean cells. Although the elicitor does not kill the soybean cells, it does stop the growth of the cells (b) and stops the uptake by the cells of ions (primarily nitrate) from the culture media (c).

The mycelial wall-released elicitor is heterogeneous in size with an average molecular weight of approximately 100,000. An exo-β-1,3-glucanase isolated from Euglena gracilis (11) hydrolyzes approximately 90% of the glucan, leaving a more highly branched fragment (Table I, column 2). The E. gracilis exoglucanase hydrolyzes the polymer from the

TABLE I. The Glucosyl Linkage Composition of Phytophthora Megasperma var. Sojae (Pms) and Yeast Elicitors

Glucosyl linkage	Pms elicitor (before exo-)	Pms elicitor (after exo-)	Yeast elicitor
	%	%	%
Terminal	17	28	23
3-linked	54	26	11
6-linked	4	10	40
4-linked	2	6	9
3,6-linked	23	30	17

The Pms elicitor composition is given both before and after exposure of the elicitor to an exo-β-1,3-glucanase isolated from Euglena gracilis. The terminal glucosyl residues are linked only through C-1. The 3-linked, 4-linked, and 6-linked glucosyl residues are linked to other glucosyl residues through C-1 and C-3, C-4, or C-6, respectively. The 3,6-linked glucosyl residues are branch points in the glucan, being linked to other glucosyl residues through C-1, C-3, and C-6.

nonreducing end and is capable of hydrolyzing the glycosidic bond of 3-linked glucosyl residues that have other glucosyl residues attached to the 6 position. The product of the exoglucanase-degraded mycelial wall-released elicitor is still size heterogenous but has an average molecular weight of approximately 10,000. This highly branched glucan fragment retains as much activity as the undergraded elicitor.

The E. gracilis enzyme hydrolyzes β-glucans which is evidence that the Pms mycelial wall glucan is a β-linked polymer. Optical rotation and NMR studies have confirmed that the glucan is β-linked. This is not surprising as other Phytophthora cell walls have a quantitatively dominant component which is a β-3-linked glucan with some branches to C-6 (12). Indeed, it appears that as much as 60% of the mycelial wall of the Pms is composed of this polymer.

Partial acid hydrolysis of the Pms cell walls releases a series of oligosaccharides which can be partially resolved by Bio-Gel P-2 gel permeation chromatography (Fig 6). The smallest oligosaccharides do not contain detectable elicitor activity, although oligosaccharides containing as few as 7 or 8 glucosyl residues are active elicitors. The area of the P-2 column containing the smallest elicitor-active oligosaccharide has been fractionated by high pressure liquid chromatography into about 5 components. Sufficient quantities of these oligomers are now being produced to permit biological and structural characterization.

Periodate treatment of the wall-released elicitor confirms the polysaccharide nature of the active component and demonstrates the essential role of a branched oligosaccharide having terminal glycosyl residues. Exposing the elicitor to periodate eliminates almost all of the elicitor activity (Fig. 7). On the other hand, if the periodate-degraded polymers are reduced with sodium borohydride and then are subjected to mild acid hydrolysis, a considerable portion of the elicitor activity is regained (Fig. 7). Since the 3- and 3,6-linked glucosyl residues (Table I) lack vicinyl hydroxyls and are, therefore, resistant to periodate degradation, it seems likely that the periodate has destroyed the elicitor activity by modifying the terminal, 6-linked, and/or 4-linked glucosyl residues of the elicitor (Table I). Recovery of elicitor activity after partial acid hydrolysis of the periodate-treated elicitor (Fig. 7) suggests that new terminal glycosyl residues have been exposed and are able to provide the proper structure of an active elicitor. The requirement for a branched oligosaccharide is supported by our observation that 3-linked glucans which lack branches to

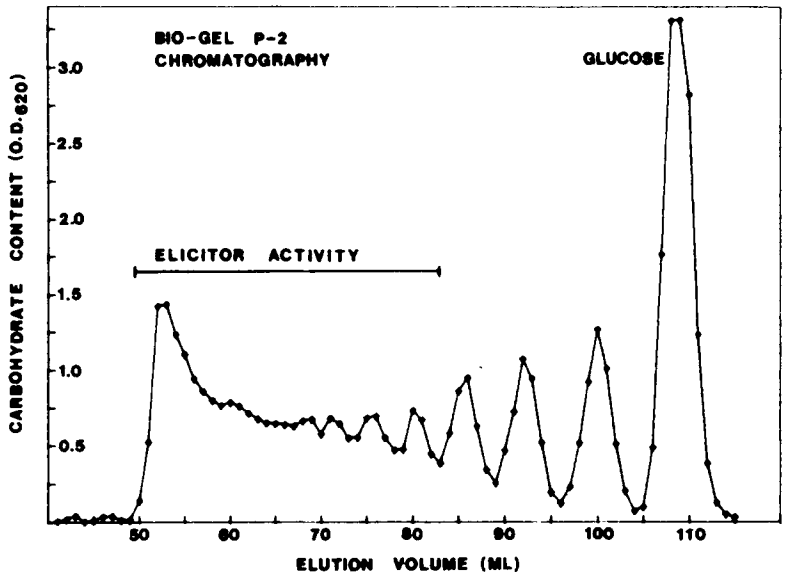

Fig. 6. Elicitor-active oligosaccharide fragments of Pms mycelial walls are generated by partial acid hydrolysis. The fragments are fractionated according to size by gel permeation chromatography. The smallest fragments possessing elicitor activity consist of 7 or 8 glycosyl residues.

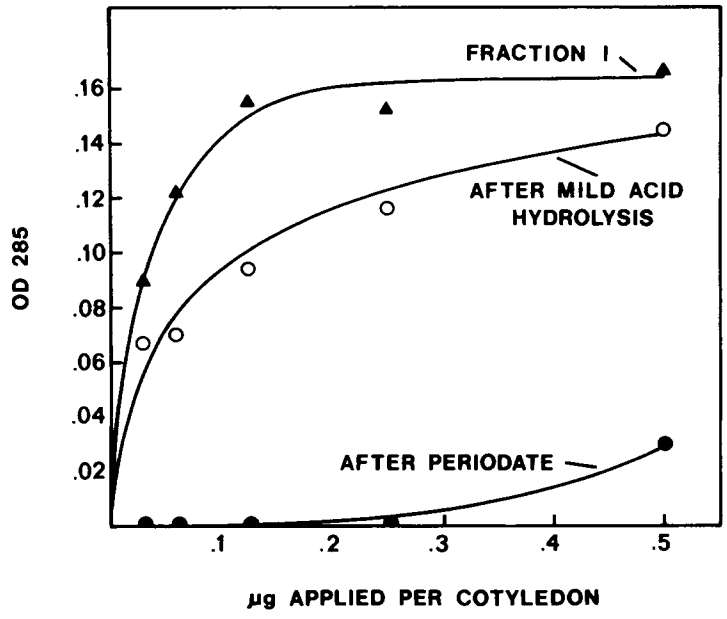

Fig. 7. Periodate (15 mM sodium metaperiodate at 20°C for 36 hr) destroys most of the Pms elicitor (Fraction I from reference 10) activity. A significant portion of this activity is recovered following reduction with sodium borohydride and mild acid hydrolysis (0.1 N H_2SO_4 at 25°C for 48 hr). The optical density at 285 nm is a measure of the amount of glyceollin accumulated in the cotyledon assay.

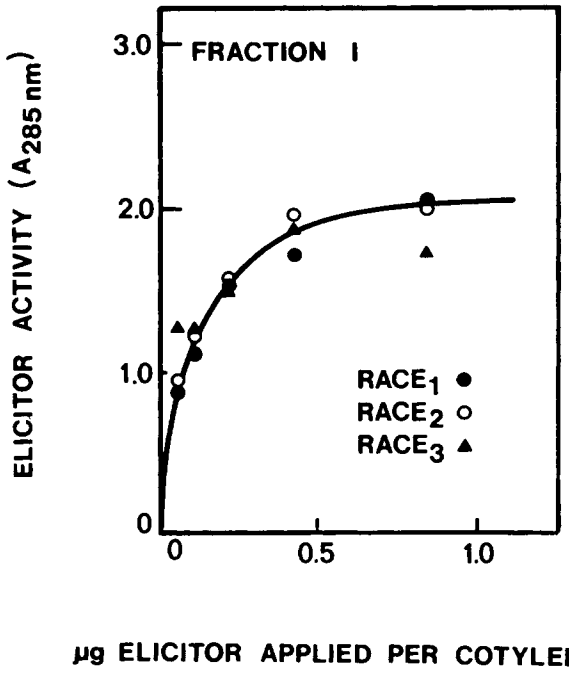

Fig. 8. The ability of the elicitors isolated from Pms races 1, 2, and 3 to stimulate glyceollin accumulation in the cotyledon assay.

C-6 or have only a single branched C-6 glucosyl residue, such as laminarin, have little or no elicitor activity (< 0.001 of the Pms elicitor).

F. Elicitors Are Widespread in Nature and Lack Species Specificity

Elicitors have been purified from 3 differentially pathogenic races of Pms and these elicitors appear structurally identical (7). Data shown on Figs. 3 and 8 demonstrate that the elicitors from the 3 Pms races have equivalent abilities to stimulate the accumulation of glyceollin soybean hypocotyls and cotyledons.

The fact that both infective and noninfective races of Pms stimulate in soybeans the accumulation of inhibitory glyceollin levels demonstrates that the accumulation of glyceollin at the observed rate is not sufficient to prevent the growth of the pathogen in susceptible cultures of soybean. This is further demonstrated in Fig. 9 in which soybeans have been exposed in the hypocotyl assay to either an incompatible race of Pms, a compatible race of Pms, or the elicitor. It can be seen that in all 3 cases it requires approximately 9 hr before inhibitory levels of glyceollin are accumulated in the plant. But the rate of glyceollin accumulation is the same whether the hypocotyls are infected with a compatible race of the pathogen, which will kill the plants within 24 hr, or an incompatible race of the pathogen, which cannot kill the plants. The fact that the purified elicitor causes the hypocotyls to produce glyceollin at the same rate as the live mycelia of both the compatible and incompatible fungi suggests that the elicitor is the mycelial component that the plant recognizes.

Soybean plants can be protected from the compatible race of the fungus by applying the elicitor to the hypocotyls 6 hr before inoculation of the hypocotyls with the mycelia

of the compatible race. The mechanism by which the compatible race of the fungus avoids the inhibitory effect of glyceollin is unknown at the present time.

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. One fungus containing such β-glucans is brewer's yeast, S. cerevisiae, a nonpathogen of plants. An elicitor has not been purified from a commercially available extract of brewer's yeast (from Difco). 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 a mannan (Table II). However, yeast extract does contain small amounts of a 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 (Table II). Both the purified mannan and glucan remain contaminated by small amounts of arabinogalactan. The ribose which contaminates the 80% ethanol insoluble fraction is removed on a DEAE-cellulose column.

The elicitor activity of the crude 80% ethanol yeast extract precipitate resides in the glucan component (Fig. 10). The small amount of residual activity remaining in the mannan fraction can be attributed to the minor contamination of this fraction by glucan. The glucan is composed of the same glucosyl linkages found in the Pms elicitor and is most similar to the Pms elicitor after degradation of the Pms elicitor by the exoglucanase (Table I). The same quantities of the yeast and Pms elicitor are required to stimulate glyceollin accumulation in soybeans.

Our laboratory has obtained other evidence that the elicitor-phytoalexin story is a general one. For example, the Pms elicitor stimulates suspension-cultured cells of sycamore and parsley to produce large amounts of phenylalanine ammonia lyase activity. In addition, we have some evidence that the Pms elicitor stimulates Phaseolus vulgaris, the true bean, to make its phytoalexins. In addition, a wall glucan from Colletotrichum lindemuthianum (13), a pathogen of P. vulgaris, stimulates soybeans to produce glyceollin. And, finally, the Pms elicitor stimulates potato tubers to accumulate their phytoalexins (Mark Wade and Peter Albersheim, unpublished results).

Thus, elicitors appear to be general in nature, and diverse plants are able to respond to a single elicitor. Elicitors may therefore provide a new way of protecting plants against their pests, for elicitors may activate the plant's own defense mechanism and thereby eliminate some of the need for spraying agricultural crops with poisonous pesticides.

II. THE LECTINS OF LEGUMES INTERACT WITH THE LIPOPOLYSACCHARIDES OF NITROGEN-FIXING RHIZOBIUM SYMBIONTS

A. Rhizobium-Lectin Interactions are Specific

Bacteria, like fungi, elicit phytoalexin accumulation in plants. Therefore, the Rhizobium symbionts of legumes must either avoid eliciting phytoalexins in their hosts, metabolize the phytoalexins when they are synthesized, or be immune to the effects of the phytoalexins. In any case, molecules exist in both the Rhizobium and their legume hosts which react in such a way that a successful symbiosis can develop. These reactions do not proceed to a successful conclusion when a Rhizobium interacts with a nonhost legume. For example, Rhizobium japonicum forms a symbiotic relationship with soybeans and must avoid being rejected by the microbial defense mechanism of soybeans. But R. japonicum does not form a symbiotic relationship with other legumes such as peas. On the other hand, R. leguminosarum forms a symbiotic relationship with peas but not with soy-

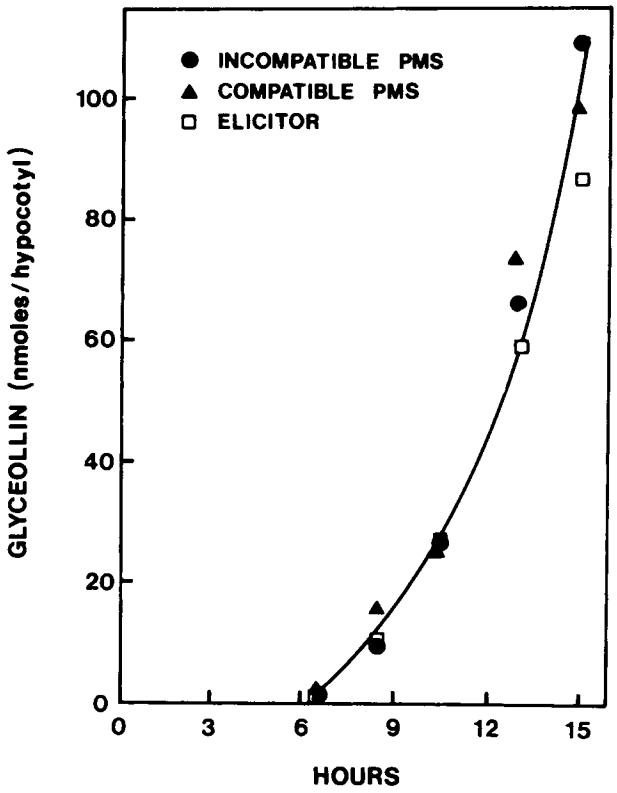

Fig. 9. Hypocotyls of soybean cultivar Harosoy 63 are stimulated to accumulate glyceollin at the same rate by incompatible Pms race 1, by compatible Pms race 3, and by Pms elicitor (isolated from either a compatible or an incompatible race).

TABLE II. The Neutral Sugar Composition of the Elicitor Obtained From Commercial Brewer's Yeast Extract (Difco)*

	Mannose	Glucose	Ribose	Arabinose	Glactose
Crude (80% EtOH Ppt)	80	8	9	2	1
Purified mannan	92	2	0	4	2
Purified glucan	2	95	0	2	1

*The values represent the % of total neutral sugar present in either the 80% ethanol precipitate of the yeast extract or in the mannan-rich fractions obtained from the 80% ethanol precipitate.

beans, and so forth. Our laboratory has been attempting to identify the molecular interactions which determine whether a particular Rhizobium-legume interaction will lead to a successful symbiosis.

B. The Lectins of Legumes Bind to the Cell Surface of Their Symbiont but not to the Cell Surface of Their Nonsymbiont Rhizobium

The lectins of legumes interact with the cell surfaces of their symbiont Rhizobium. Bohlool and Schmidt (14) were the first to provide evidence for this phenomenon. These

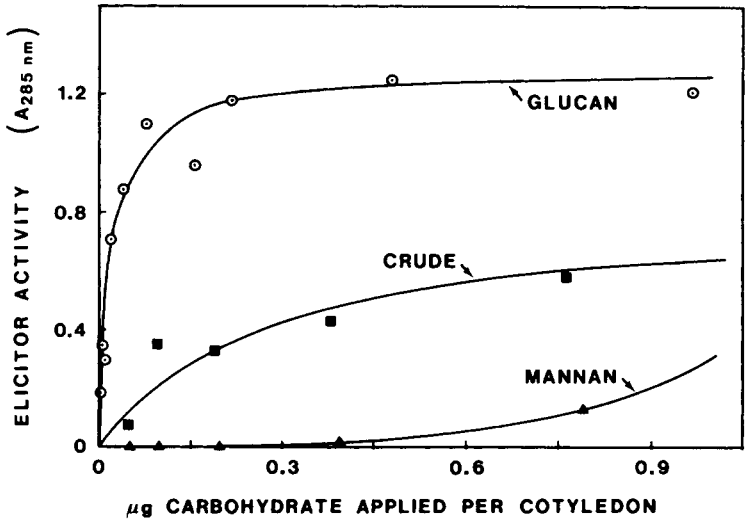

Fig. 10. The ability of the 80% ethanol precipitate of "crude" yeast extract to stimulate glyceollin accumulation in soybean cotyledons is compared with the same abilities of glucan-rich and mannan-rich fractions isolated from the "crude" yeast extract.

workers extracted soybean seed lectin and conjugated it with a fluorescent dye. They found that this fluorescence-labeled soybean lectin preparation was bound to all but 3 of 25 strains of R. japonicum, the symbiont of soybeans. They found, too, that the fluorescent-labeled lectin did not bind to any of 23 other strains representative of Rhizobium species which do not nodulate soybeans. The laboratories of W. Dietz Bauer at the Charles F. Kettering Research Institute and Jack Paxton at the University of Illinois have confirmed this basic observation (unpublished personal communication). These laboratories have found that more highly purified soybean lectin binds to about half of the symbiont R. japonicum strains, but they found no binding of soybean lectin to Rhizobium strains which do not nodulate soybeans.

C. The Lectins of Legumes Interact With the Lipopolysaccharides of Their Symbiont but Not With the Lipopolysaccharides of Their Nonsymbiont Rhizobium

The dominant surface antigens of the Gram-negative Rhizobium are the O antigens of the lipopolysaccharides. Our laboratory has asked whether legume seed lectins interact with the Rhizobium lipopolysaccharides. Affinity-purified lectins from the seeds of 4 legumes were covalently attached to Agarose by cyanogen bromide coupling. The lipopolysaccharides were purified from the 4 Rhizobium symbionts of the legumes from which the lectins were purified. The exopolysaccharides were also purified from each of the 4 Rhizobium species. The lipopolysaccharides and the exopolysaccharides were passed separately through columns containing the legume lectins bound to Agarose. It was then determined whether any of the lipopolysaccharides or exopolysaccharides were retained by the lectin columns. The lipopolysaccharides isolated from nonsymbiont Rhizobium do not bind to the lectin columns. Neither do the exopolysaccharides bind to the lectin columns, regardless of whether the exopolysaccharides were synthesized by symbiont or nonsymbiont Rhizobium. The inability of pea lectin to bind lipopolysaccharides and exopolysaccharides produced by R. japonicum, R. phaseoli, and R. spp. is illustrated by the open circles and dotted lines of Fig. 11. The pea lectin column also does not bind the exopolysaccharide of

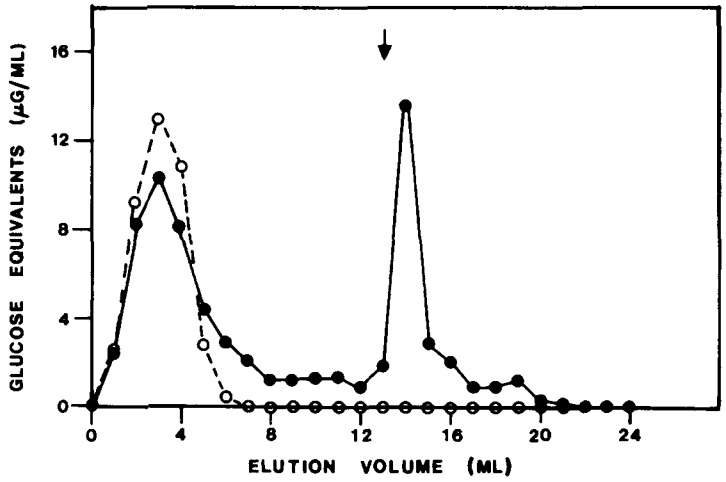

Fig. 11. The exopolysaccharides of Rhizobium japonicum, R. phaseoli, R. spp. and R. leguminosarum and the lipopolysaccharides of the first 3 of these Rhizobium species pass directly through an affinity column composed of pea lectin covalently attached to Agarose (open circles, dashed line). The lipopolysaccharide of R. leguminosarum interacts with the pea lectin (filled circles, solid line); a portion of the lipopolysaccharide is released when the column is washed (arrow) with pH 3 buffer. Peas are the symbiont host of R. leguminosarum. The polysaccharides are detected as glucose equivalents by the anthrone assay.

R. leguminosarum. But the pea lectin column does bind at least a portion of th lipopolysaccharide produced by R. leguminosarum (filled circles of Fig. 11). Thus, since R. leguminosarum is the symbiont of peas, the pea lectin binds partially the lipopolysaccharide of its symbiont Rhizobium but does not bind the lipopolysaccharides of 3 nonsymbiont Rhizobium. The data of Table III summarize the results obtained for the interaction of the 4 lipopolysaccharides and the 4 lectin columns (15). In every case, the lipopolysaccharides interact with their host-legume lectin, but not with the lectins from non-host legumes.

The binding between the lipopolysaccharides and the lectin appears to be relatively weak; continued washing of the columns with pH 7 buffer eventually washed off most of the bound lipopolysaccharide. However, as illustrated in Fig. 11, the material remaining on the column after limited washing with pH 7 buffer is immediately washed off the column with pH 3 buffer, a treatment which characteristically negates lectin binding.

D. Are Lectins Enzymes?

We are not certain why the lipopolysaccharides do not bind completely to their host-legume lectins. The amount of lipopolysaccharide which binds to the lectin column depends on the time the lipopolysaccharide interacts with the column. Indeed, there appears to be an optimal time for interaction; as illustrated on Fig. 12, more lipopolysaccharide binds to the column when allowed to react with the column for 100 min rather than for the few minutes required for the lipopolysaccharide to pass directly through the 3-ml bed volume affinity column. On the other hand, the amount of lipopolysaccharide which binds decreases again after even longer interaction times. This and other results suggest that a chemical reaction may be occurring on the lectin columns. Perhaps the

TABLE III. Interaction of Rhizobium Lipopolysaccharide (LPS) and Exopolysaccharide With Immobilized Lectins*

Rhizobium species and component	Source of immobilized lectin			
	Soybean	Pea	Red kidney bean	Jack bean
R. japonicum LPS	35	0	0	0
R. leguminosarum LPS	0	36	0	0
R. phaseoli LPS	0	0	5–23	0
R. spp. LPS	0	0	0	25
Exopolysaccharide from each of the above species	0	0	0	0

*The data represents the percentage of the anthrone positive lipopolysaccharides material which did not pass directly through the affinity column and which was eluted at pH 3.0 (15).

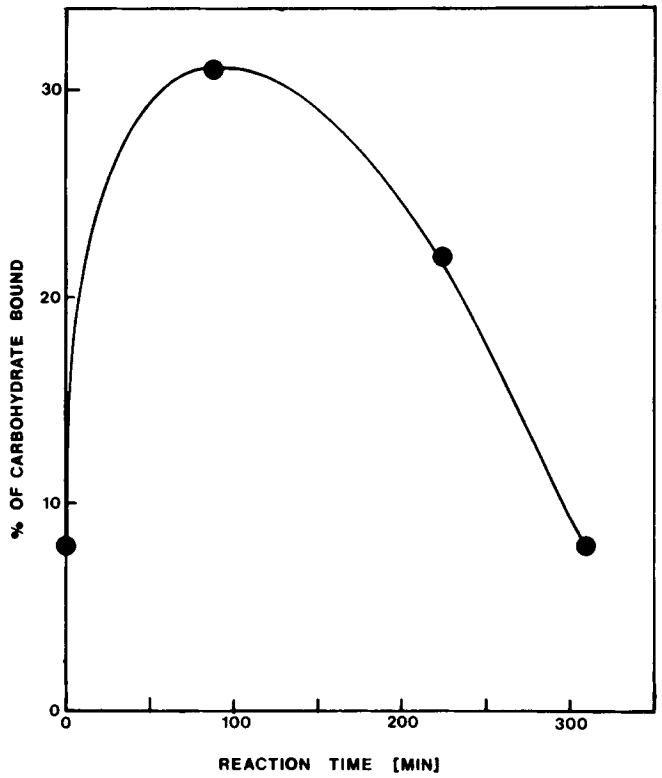

Fig. 12. The degree of binding of the Rhizobium lipopolysaccharides to their host-legume lectin columns (Fig. 11 and Table III) depends on the length of time the lipopolysaccharide interacts with the lectin column.

lectins or contaminants of the lectins might be interacting with the lipopolysaccharides of their symbiont Rhizobium in such a way that the lectins are actually altering the lipopolysaccharides. In other words, some component of the lectin columns might be catalyzing a reaction with the lipopolysaccharides.

We have tried very hard, without total success, to demonstrate whether or not lectins are enzymes. The pea lectin column does appear to alter the size of the lipopolysaccharide from R. leguminosarum (the pea symbiont), but the pea lectin does not alter the size of the lipopolysaccharides from several other Rhizobium species. The phytohemagglutinin (kidney bean lectin) column specifically alters the size of the R. phaseoli (the kidney bean

symbiont) lipopolysaccharide. However, it is not safe to assume that the apparent change in size of the lipopolysaccharides is an enzyme-catalyzed alteration as the observed reaction rate was slow and the apparent size of lipopolysaccharides is highly dependent on the degree of micelle formation. Perhaps the size change resulted from a breakdown of the micelles.

Additional experiments were carried out and provided some evidence that the structure of the R. phaseoli lipopolysaccharide was being altered by interaction with the phytohemagglutinin column. These experiments depended on the fact that the untreated lipopolysaccharide of R. phaseoli binds completely to DEAE-cellulose. After interaction with the phytohemagglutinin column, approximately 50% of the carbohydrate of the lipopolysaccharide from R. phaseoli no longer binds to the DEAE-cellulose column. However, this result could also stem from a change in the degree of micelle formation of the lipopolysaccharide. And again, the rate of the observed reaction was slow. These reactions could only be demonstrated using the large amount of lectins which are covalently attached to the affinity columns. Smaller amounts of soluble lectins did not give reproducible results.

It may not be the lectins which are catalyzing the apparent reactions, but even if other proteins in the lectin preparations are responsible, these proteins must retain the observed host-symbiont specificity, for the putative catalytic factors are only capable of altering the lipopolysaccharide of the Rhizobium which are symbionts of the legumes from which the catalytic factors were isolated.

The possibility that lectins are, in fact, enzymes did not occur to us before our studies of the interactions between lectins and lipopolysaccharides. However, it might have occurred to us had we been aware of the work of Scheid and Choppin (16) who demonstrated that the larger of the 2 coat glycoproteins of paramyxovirus SV5 and the Newcastle disease virus are both a neuraminidase and a hemagglutinin. We would have been even less surprised had we been aware of the papers of Rood and Wilkinson (17, 18) who obtained a great deal of evidence that a protein secreted by Clostridium perfringens is both a sialidase and a hemagglutinin. It seems probable that lectins agglutinate animal cells because they are interacting with pseudo-substrates or perhaps one of a pair of substrates. Any enzyme involved in carbohydrate metabolism and which has 2 active sites can act as a lectin. We would imagine that under certain conditions, most if not all glycosyl transferases which have at least 2 active sites, would act as lectins. It might be that the observed relatively poor binding constants of lectins for their haptens is simply a reflection that the correct substrates have not been identified. Our biased opinion is that lectins will be found to serve many functions in plants and that many lectins are enzymes. Much more evidence will have to be obtained before this suggestion can be considered to be proven. Nevertheless, those using lectins in their studies should consider the possibility that lectins have catalytic activity.

E. The Lipopolysaccharides of Each Species of Rhizobium and Each Strain of Each Species Are Structurally Unique

If the lectins of legumes interact with the lipopolysaccharides of symbiont Rhizobium and not with the lipopolysaccharides of nonsymbiont Rhizobium, then different Rhizobium species must have different lipopolysaccharides. Carbohydrate analysis of the lipopolysaccharides purified from several Rhizobium species and from 2 or more strains of each of the species demonstrates that not only are the lipopolysaccharides of the different Rhizobium species structurally unique, but the different strains of the various Rhizobium species are structurally unique (Table IV).

TABLE IV. The Lipopolysaccharide Sugar Composition of Several Strains of Each of Three Rhizobium Species*

	R. phaseoli		R. leguminosarum			R. japonicum		
	Strain		Strain			Strain		
	127K17	127K24	128C53	3HOQ1	128C63	61A23	61A135	R54a
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+
Uronic acids	+	+	+	+	+	+	+	+
KDO	+	+	+	+	+	±	±	±
Fucose	+	+	+	+	+	+	—	+
Rhamnose	—	—	+	+	—	+	—	+
2-O-Me-6-dode-oxyhexose	—	+	—	—	—	—	—	—
3-N-me-amino-3,6-dodeoxyhexose	+	—	—	—	+	—	—	—
2-Amino-2,6-dode-oxyhexose (1)	+	—	—	—	—	—	—	±
2-Amino-2,6-dode-oxyhexose (2)	—	—	—	—	—	—	—	±
Di-O-me-6-deoxyhexose	—	—	—	—	+	—	—	—
6-O-Me-hexose	—	—	—	—	—	+	—	—

+ = sugar present in the lipopolysaccharide.
± = sugar may be present
— = sugar is not present

The lipopolysaccharides of the Rhizobium strains that we have studied are composed of sugars which are typical of the sugars which compose the lipopolysaccharides of other Gram-negative bacteria (19). Not only do these lipopolysaccharides have a diversity of sugars, but the lipopolysaccharides are characterized by the presence of rather unusual sugars such as 3-O-methyl-3-amino-3, 6-dideoxyhexoses or 2-O-methyl-6-deoxyhexose. As in other Gram-negative lipopolysaccharides, the Rhizobium lipopolysaccharides also contain 2-keto-3-deoxyoctanoic acid, a characteristic carbohydrate of the core region of Gram-negative lipopolysaccharides.

Certain sugars are found in all of the Rhizobium lipopolysaccharides examined to date. Some of all of these may be part of the core regions of the lipopolysaccharides. Other sugars are found in only one or a few different lipopolysaccharides, and these may be characterisitic of the O-antigen regions of the lipopolysaccharides. One quite common component of the Rhizobium lipopolysaccharides, not frequently reported for other Gram-negative bacteria, is galacturonic acid.

Immunological studies also indicate that the lipopolysaccharides of different strains of a Rhizobium species have different structures. The lipopolysaccharides of 2 R. leguminosarum strains have the same chamical compositions, or, in other words, the same chemotypes. But even these strains can be differentiated immunologically. For example, the lipopolysaccharide from R. phaseoli strain 127K17 was used to raise antibodies in a rabbit. These antibodies, when placed in the center well of a semimicro double diffusion plate form a precipitin band when run against the lipopolysaccharide or whole cells of

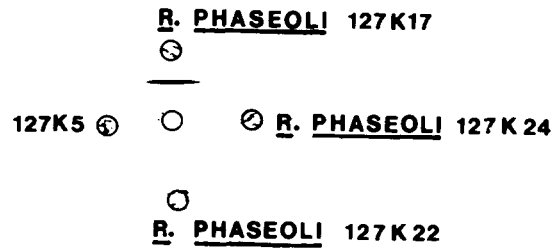

Fig. 13. Rabbit antibodies raised against the lipopolysaccharide isolated from R. phaseoli strain 127K17 were placed in the center well of this semimicro double diffusion gel. The outer wells contained frozen-dried cells (or isolated lipopolysaccharides, not shown) of the R. phaseoli strains indicated. A precipitin band only forms between the antibodies and the R. phaseoli cells of the same strain from which the lipopolysaccharide was isolated and used to raise the antibodies.

R. phaseoli 127K17 (Fig. 13). But the antibodies to R. phaseoli 127K17 do not react with whole cells of the lipopolysaccharides of the 3 other R. phaseoli strains illustrated in Fig. 13 or 3 other R. phaseoli strains not present in Fig. 13. In addition, the antibodies raised against the lipopolysaccharides of R. japonicum 61A23 react with the lipopolysaccharides of that R. japonicum strain but not with 2 other strains of R. japonicum nor with several other Rhizobium species. The specificity of the lipopolysaccharide antibodies is equally apparent in agglutination assays. Thus, the lipopolysaccharides of different Rhizobium strains have unique structures.

Since the lectins of legumes interact with the lipopolysaccharides of their symbiont Rhizobium, the legumes must interact with the different lipopolysaccharides originating from the different strains of a single Rhizobium species. If this is true, the lectins may be interacting with a common portion of the O antigens of these lipopolysaccharides or else the lectins may be interacting with the core regions of the lipopolysaccharides of their symbiont Rhizobium. We have obtained some evidence that the core regions of different Rhizobium species are in fact structurally different. This would suggest that the Rhizobium species might actually be considered to represent different genera and that the different strains of a Rhizobium species deserve species rank. Regardless of the proper classification of the Rhizobium, it is clear that the lipopolysaccharides contain enough structural information to account for the specificity of host-symbiont selection.

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