An Inducer of Soybean Phytoalexin and its Role in the Resistance of Soybeans to Phytophthora Rot

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ABSTRACT

A material capable of inducing phytoalexin production in soybean plants was isolated from *Phytophthora megasperma* var. *sojae*. This inducer is soluble in methanol, and has a paper chromatographic R_F of 0.23 using a butanol:acetic acid:water solvent (4:1:5, v/v). The molecular weight of the inducer is in the range of 10-30,000, and chemical tests indicate that it is a glycoprotein.

The production of this inducer is the key reaction attributed to the *Rps* gene for resistance in soybeans. The amount of inducer produced by the fungus was greatly increased when the fungus was placed in contact with the resistant (Harosoy 63) host tissue for 8 hr. This same increase in the amount of inducer could be stimulated by placing the fungus in the filter-sterilized juice of the resistant host for 8 hours. When the same procedures were carried out using the susceptible (Harosoy) host as the stimulator, there was no increase in the amount of inducer present in the fungus. Other soybean varieties carrying the *Rps* gene reacted in a manner similar to the resistant Harosoy 63 variety. Phytopathology 61:954-958.

Additional key words: disease resistance.

Phytoalexins play a prominent role in the defense mechanism of the soybean plant against *Phytophthora* root rot (3, 6, 9). The pathogen, *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildeb., race 1 (Pms_1), has been generally studied on two soybean varieties, Harosoy (susceptible) and Harosoy 63 (resistant). A single dominant gene conditions the inheritance of resistance in the variety Harosoy 63 (1). To date, all resistant varieties have the Mukden, single-dominant-gene resistance, and the gene has been designated *Rps* (8). The variety Harosoy 63, which has this genotype, produces a yellow, green fluorescing phytoalexin (PA_k) within 4 hr after infection by Pms_1, and this production increases with time until the pathogen is killed (6). The susceptible Harosoy variety is nearly identical to the resistant variety, but does not have the *Rps* gene. It produces PA_k within 4 hr after infection by Pms_1, but the PA_k disappears after 8 hr. The ability of Harosoy to produce this PA_k when infected with the soybean pathogen and also when inoculated with nonpathogens of soybean (7, 9) indicates that the *Rps* gene is not directly responsible for PA_k production.

One possible function of the *Rps* gene might be the induction or triggering of the PA_k production after invasion by Pms_1. From the fungus *Monilinia fructicola,* Cruickshank (5) isolated an inducer of the phytoalexin produced by french beans (phaseollin). The inducer (Monilicolin A) was a polypeptide with a molecular wt of 8,000.

This study was undertaken to determine whether an inducer of PA_k could be isolated from *P. megasperma* var. *sojae,* to study its possible role in the mechanism of resistance, and to determine the possible role of the *Rps* gene in governing disease resistance in the soybean.

MATERIALS AND METHODS.—Two soybean varieties, Harosoy (susceptible to Pms_1) and Harosoy 63 (resistant to Pms_1) were used in most experiments. These varieties were designated as H_S and $H63_R$, respectively, throughout this paper. All soybean plants used in the following experiments were grown for 7-10 days in a greenhouse sand bench at 23 C and natural photoperiod.

The soybean pathogen Pms_1 was grown in V-8 juice broth or soybean broth (9) for 10 days in all experiments. The V-8 juice broth was prepared by mixing 200 ml filtered V-8 juice, 800 ml water, and 2 g $CaCO_3$. The cultures were incubated as still cultures at room temperature in 250-ml flasks, each containing 100 ml of broth.

Bioassay for PA_k inducer.—Cotyledons were removed from 7- to 10-day-old $H63_R$ soybean plants, immersed for 5 min in a 0.25% sodium hypochlorite solution, and then placed under running water for 1 hr to remove all traces of sodium hypochlorite. The cotyledons were aseptically prepared for bioassay by slicing off the epidermis and a portion of the mesophyll cells from the lower side to form a cup-shaped depression.

Since the physiological condition of the cotyledons influenced their response to the inducer, the mesophyll tissue was carefully examined after slicing. When the tissue was yellow and granular in appearance, the cotyledon was immature. When the tissue was flaccid and green with yellow mottling, the cotyledon was senescent and gave a poor response. The cotyledons with mesophyll tissue of a uniform, light green color gave maximum response to the inducer.

Three ml of sterile water were placed in a petri dish and five-six cotyledons were floated in the dish with the incised depression facing upward. The solutions to be tested for presence of PA_k inducers were carefully layered in the incised cotyledon depressions to form a large drop. The cotyledons in covered dishes were then exposed to fluorescent light for 24 hr. When PA_k inducers were present, a dark red color developed in the plant tissue and any remaining liquid.

Isolation and stimulation of a phytoalexin inducer.—After 10 days' growth, fungus mycelium was removed by filtration from 100 V-8 broth flasks. The broth designated as the culture filtrate was centrifuged and bioassayed for PA_k inducer. The mycelium was suspended in deionized water for 1 hr, then rinsed 5 times with deionized water. The water residues were pooled and added to the filtrate sample to be bioassayed. The mycelium was then ground with quartz sand in a mortar, 20 ml of deionized water were added to the mycelium, and the mixture was centrifuged (12,000 *g* for 30 min). The supernatant was collected, the mycelium resuspended in 20 ml of water, and this procedure was repeated 3 times. The resultant supernatant was labeled mycelial extract, adjusted to 100 ml in volume, and bioassayed. The remaining mycelial fragments were also bioassayed. This same procedure was followed with the pathogen grown in soybean broth. Soybean and V-8 juice broth were bioassayed as controls. All fractions bioassayed were filter-sterilized.

To study the effect of the host on induced production, 100 $H63_R$ plants were inoculated with uniform pieces of Pms_1 mycelium (10-day-old cultures). A small wound was made on the plant hypocotyl ca. 5-10 mm below the cotyledonary node, and a piece of mycelium was inserted into the wound. After 8 hr, the mycelium was removed from each wound and extracted. The extract volume was adjusted to 100 ml and bioassayed. This same procedure was repeated with mycelium removed from H_S plants.

One hundred g of 7-day-old $H63_R$ soybean hypocotyls were ground at room temperature in a blender with 500 ml of water. The slurry was strained, centrifuged, and Millipore-filter-sterilized to be used as inducer-stimulating juice. Ten 10-day-old fungal cultures were washed with sterile water and placed in the sterile juice for 8 hr. This procedure was repeated with the autoclaved fungus, autoclaved $H63_R$ juice, and autoclaved and filter-sterilized H_S juice. The stimulated mycelium was removed from the juice after 8 hr in all experiments, extracted as described above, and called juice-stimulated extract. The extract was sterilized before bioassay by both filtration and autoclaving. The filtered soybean hypocotyl juice was adjusted to 100 ml and called stimulated filtrate. H_S and $H63_R$ juice served as nonstimulated controls, and all samples were bioassayed.

Quantitative inducer studies.—The approximate quantity of inducer present or stimulated in the pathogen was determined indirectly by measuring the amount of PA_k induced. The previously described bioassay procedure was used with the following modifications. Two equal wounds were made with a No. 2 cork-borer to a depth of 2 mm on the lower side of the $H63_R$ cotyledons. Stimulated and nonstimulated filtrates and extracts were placed in these wounds for 24 hr. The H_S and $H63_R$ extracts and filtrates were obtained from 100 V-8 broth cultures. The juice-stimulated extracts and filtrates were obtained from 10 V-8 broth cultures. Each sample was adjusted to 100 ml and tested, then concentrated to 10 ml and retested. Each sample was placed into 10 wounds (5 cotyledons), and after 24 hr the 5 cotyledons were placed in a beaker containing 10 ml of water. The beakers were placed on a shaker for 3 hr, the cotyledons were discarded, and the samples were measured spectrophotometrically at 489 nm where PA_k has intense absorption at pH 7. The amount of PA_k induced was checked by comparison with a relatively pure 4 μg/ml sample.

Four genetically different soybean varieties were used to study the relationship between the *Rps* gene and inducer production. The varieties were Illini and Blackhawk, resistant to Pms_1; and Hawkeye and Capitol, susceptible to Pms_1 (10). Sterile plant juice of these varieties was prepared and tested for inducer formation similarly to the juice from $H63_R$.

Purification and characterization of the inducer.—An attempt was made to partially characterize the inducer. Eighty V-8 broth flasks of 10-day-old Pms_1 were used to obtain a large quantity of inducer. The mycelium was washed, stimulated for 8 hr, and bioassayed for inducer formation. The active extract was adjusted to 100 ml and dialyzed against deionized water for 3 days with water changes every 24 hr, and each water solution was bioassayed for activity. The water solutions outside the dialysis bag were concentrated, combined, and extracted 6 times with chloroform and 3 times with diethyl ether. The aqueous phase was dried on a flash evaporator and extracted 3 times with absolute methanol. The methanol extracts were pooled, dried, resuspended in deionized water, and bioassayed. The methanol-extracted sample was chromatographed on a Sephadex G-10 column and after the void volume, 5-ml aliquots were collected. The aliquots were bioassayed for the active fraction, and the procedure was repeated with Sephadez G-50 and G-75 columns. The active fraction was chromatographed on Whatman 3MM paper using the organic phase of a butanol:acetic acid:water solvent (4:1:5, v/v). The paper was cut horizontally into 1-inch strips which were eluted and bioassayed. The active fraction was again spotted on 3MM paper and the above procedure repeated. The active fraction was subjected to paper electrophoresis for 3 hr (pH 7.0, 500 v, 1 M K_2HPO_4 buffer). The paper was then cut into 1-inch strips which were eluted and bioassayed. The active fraction was analyzed on an ultraviolet spectrophotometer, acid hydrolyzed in 2 N and 8 N HCl, and the active fraction analyzed for sugars using a Molisch test (4), aniline-acid-oxalate test (4), and Nelson's test (4). The active fraction was analyzed for proteins using a biuret test (4), 8 N hydrochloric acid hydrolysis followed by ninhydrin and Folin-Ciocalteau tests (4). Sudan black was used for a general lipid test (4). The active fraction was chromatographed on a Corning CPG-10, 75A glass bead column in water to determine the approximate molecular wt. These same procedures were carried out with nonstimulated fungus cultures.

Inducer production by other fungi.—Several different fungal cultures (grown on V-8 broth) were stimulated as previously described, extracted, and checked for inducer production. The fungi tested were: *Venturia*

inaequalis, Rhizoctonia solani (from soybean), *Diplodia zeae, Helminthosporium turcicum, Neurospora crassa,* and a *Penicillium* sp. All of the spore-producing organisms were cultured on V-8 agar plates for 2 weeks and the spores removed by washing with sterile water. The spores were stimulated with $H63_R$ juice for 8 hr, extracted as previously described, and bioassayed.

RESULTS.—*Isolation and stimulation of a phytoalexin inducer.*—The mycelial extract induced a positive response on the soybean cotyledons in all tests, and when the red droplets on the cotyledons were checked spectrophotometrically, the presence of PA_k (6) was confirmed. The culture filtrate showed little or no activity in all tests. The water checks showed no activity. Mycelial extracts from V-8 cultures produced some inducer activity, due to the superior mycelial growth in the V-8 broth. V-8 broth, mycelial fragments, and soybean broth did not induce a positive response in the bioassay.

The extract obtained from mycelium which had been placed in hypocotyls of $H63_R$ induced much more PA_k than the extract from mycelium placed in hypocotyls of H_S or the extract obtained from 100 V-8 broth flasks. This indicated that inducer production increased when the fungus was in contact with the resistant plant. The 10-day-old mycelium from V-8 broth which normally showed little inducer activity gave a strong, positive response after stimulation with the $H63_R$ juice, and the residual $H63_R$ juice also showed inducer activity. The autoclaved fungus did not give a positive inducer response after it was removed from the $H63_R$ juice. The H_S juice-stimulated extracts or filtrates also induced little or no PA_k. Autoclaved and nonautoclaved H_S or $H63_R$ juice did not stimulate any PA_k production. A positive response was obtained with autoclaved extracts of the fungus, which indicated the inducer is relatively heat stable.

Quantitative inducer studies.—The relative amounts of PA_k induced by fungal extracts and filtrates were calculated and presented in Table 1. When inducer samples were tested at the 100-ml concentration, only the $H63_R$ juice-stimulated samples gave positive results. If the inducer sample was concentrated to 10 ml, the H_S-stimulated samples and nonstimulated extracts gave weak positive reactions.

TABLE 1. Relative amounts of phytoalexin (PA_k) induced by extracts of stimulated and nonstimulated mycelium culture filtrates, and controls

Sample tested	Amt PA_k (ng)	
	100-ml Sample	× 10 Conc
Water control	0	0
Broth control	0	0
Fungal extract	0	21
Culture filtrate	0	0
Harosoy 63_S—Stimulated extract	0	27
Harosoy 63_S—Stimulated filtrate	0	20
Harosoy 63_R—Stimulated extract	170	750
Harosoy 63_R—Stimulated Filtrate	45	430

TABLE 2. Relative amounts of phytoalexin (PA_k) induced by *Phytophthora megasperma* var. *sojae* after stimulation by four different soybean varieties

Soybean variety	Amt PA_k (ng)	
	100-ml Sample	× 10 Conc
Illini (resistant)	97	520
Blackhawk (resistant)	129	640
Hawkeye (susceptible)	0	33
Capitol (susceptible)	5	72

The results obtained with the four different soybean varieties tested for stimulatory activity are summarized in Table 2. The two resistant varieties carrying the *Rps* gene for resistance in different genetic backgrounds gave results similar to the $H63_R$ variety. There was a slight variation among the susceptible varieties, since Capitol stimulated some inducer production.

Purification and characterization of the inducer.—The dialyzed fraction of the stimulated mycelial extract gave positive results in the bioassay. The majority of the inducer was dialyzed out within 24 hr. Chloroform and diethyl ether extractions of the dialyzed solution gave no activity, but the absolute methanol extraction removed the activity from the dried sample. The active fraction appeared in the void volume with Sephadex G-10 and G-50 and appeared within 15 ml after the void volume with Sephadex G-75. The R_F value of the active fraction in BAW was 0.23, and the material did not fluoresce under ultraviolet light. The compound had a slight positive charge by electrophoresis at pH 7.0, and had a small ultraviolet absorption peak at ca. 278 nm. A positive test for sugars was obtained before and after 2 N hydrolysis, and the Nelson's test confirmed the presence of a reducing sugar. The biuret test was positive before 8 N hydrolysis, and the ninhydrin test was negative. After 8 N hydrolysis, the reverse was true. Chromatography on the CPG-10 column indicated that the molecular wt of the compound was in the range of 10-30,000. After 2 N or 8 N hydrolysis, the compound was no longer capable of PA_k induction. It appears that the inducer is a glycoprotein, but further tests are necessary to confirm its composition.

Inducer production by other fungi.—The saprophytic fungi, *Neurospora* and *Penicillium,* did not induce any PA_k with mycelium or spore extracts. All plant-pathogenic fungi induced some PA_k. The mycelium of the soybean pathogen, *Rhizoctonia,* was capable of inducing PA_k. This organism does not produce any spores. *Helminthosporium, Diplodia,* and *Venturia* are not pathogenic on soybean plants. Their spore extracts induced PA_k, but their mycelial extracts did not.

DISCUSSION.—The role of the *Rps* gene in the total disease resistance mechanism of the soybean plant is related to the production of PA_k. The saprophytic fungi tested did not induce PA_k, even after stimulation in juice from the resistant plant. The plant-pathogenic fungi were able to induce PA_k in all experiments, depending on whether spores or mycelium were tested. When the general mode of plant invasion was spore germination, followed by penetration of the host by a germ tube, the spores were able to induce PA_k. The actual mechanism that distinguishes pathogen from nonpathogen is yet to be determined.

The fact that plant-pathogenic fungi are able to induce PA_k in soybeans is now well established. The production of PA_k which is fungitoxic may prevent invasion, thus classifying the fungi as nonpathogenic on soybean. H_S can produce amounts of phytoalexin equal to that produced by $H63_R$ (7). In susceptible plants, the soybean pathogen, *P. megasperma* var. *sojae,* has by some means lost the capacity to induce PA_k in large amounts, or can tolerate the small amount of PA_k induced in the first 4 hr after inoculation. The development of soybean varieties with the *Rps* gene enabled this pathogen to again induce PA_k and provoke the resistant response. The *Rps* gene is related to PA_k induction and is not directly responsible for PA_k production.

As a result of various chemical tests, the inducing substance appears to be a glycoprotein, but further chemical tests are needed for confirmation of this. Studies to determine whether the inducers from other plant pathogens are similar to the *P. megasperma* var. *sojae* inducer are presently in progress.

We propose the following scheme (Fig. 1) as the possible mechanism of resistance in soybeans to *Phytophthora* rot, controlled by the *Rps* gene. The host

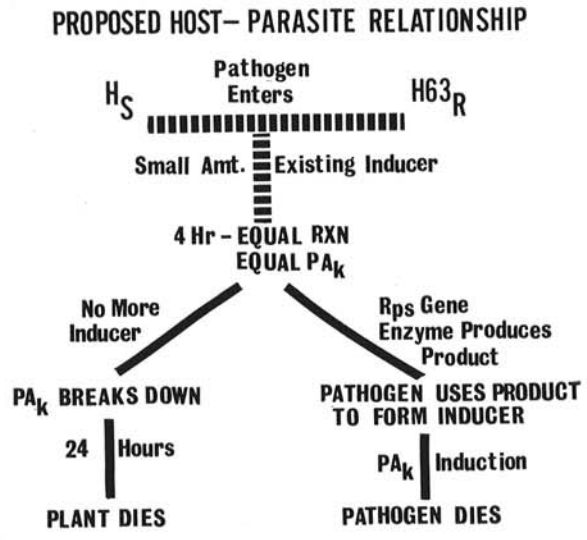

Fig. 1. Schematic diagram of the proposed host-parasite relationship among *Phytophthora megasperma* var. *sojae,* race 1, Harosoy, and Harosoy 63 soybean plants.

reactions in the first 4 hr are similar in the H_S and $H63_R$ varieties, as is the amount of PA_k produced (6). This PA_k could be triggered by the small amount of inducer already present in the pathogen. At low PA_k levels, there is little or no detrimental effect to the pathogen. Once the existing inducer has been exhausted in the H_S plant, the production of PA_k stops and the PA_k that was produced breaks down. The pathogen continues to invade the plant, and often within 24 hr the plant hypocotyl may collapse.

The $H63_R$ variety with the *Rps* gene for resistance produces PA_k within 4 hr after infection, and this production increases with time. The *Rps* gene, triggered in some way by the invading pathogen, produces a product (possibly by means of an enzyme). This product could be a direct precursor to the inducer itself, but the mechanism from gene action to inducer stimulation is yet to be resolved. An enzymatic system is proposed, since the system is heat-labile both in the living plant (2) and in the $H63_R$ juice used to stimulate inducer production. The product of this enzymatic reaction is utilized by the pathogen to produce more inducer. Once the additional inducer is stimulated, PA_k is produced and the inducer stimulation continues as long as the pathogen remains viable. Once the pathogen is killed (within 72 hr), inducer stimulation is no longer possible or necessary, and the existing PA_k breaks down.

The proposed mechanism above relates only to the soybean plant and the *Rps* gene. The induction of PA_k could well be the basis of hypocotyl resistance in young soybean plants to other pathogens. The resistance in soybean leaves to fungal invasion has not been studied, and the mechanism involved might be totally unrelated to that in the hypocotyl. Once the mechanism by which the *Rps* gene actually stimulates the inducer is known, the complete host-parasite relationship between the soybean and *P. megasperma* var. *sojae* may be determined. This knowledge in turn could shed some light on the complex nature of disease resistance in general.

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