

Host-Pathogen Interactions

XV. FUNGAL GLUCANS WHICH ELICIT PHYTOALEXIN ACCUMULATION IN SOYBEAN ALSO ELICIT THE ACCUMULATION OF PHYTOALEXINS IN OTHER PLANTS¹

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ABSTRACT

A β -glucan isolated from the mycelial walls of *Phytophthora megasperma* var. *sojiae* and a glucan purified from yeast extract stimulate the accumulation of phytoalexins in red kidney bean, *Phaseolus vulgaris*, and stimulate the accumulation of the phytoalexin, rishitin, in potato tubers, *Solanum tuberosum*. These glucans have previously been shown to be potent elicitors of glyceollin accumulation in soybean, *Glycine max*.

Treatment of kidney bean cotyledons with the glucan elicitors resulted in the accumulation of at least five fungistatic compounds. These compounds migrate during thin layer chromatography identically to the fungistatic compounds which accumulate in kidney beans which have been inoculated with *Colletotrichum lindemuthianum*, a fungal pathogen of kidney beans.

Potatoes accumulate as much as 29 micrograms of rishitin per gram fresh weight following exposure to the glucan from *Phytophthora megasperma* var. *sojiae* and as much as 19.5 micrograms of rishitin per gram fresh weight following exposure to yeast glucan. Potatoes accumulated 28 micrograms of rishitin per gram fresh weight following inoculation with live *Phytophthora megasperma* var. *sojiae*.

Many plants respond to invasion by both pathogenic and nonpathogenic microorganisms by producing phytoalexins (12, 17, 19, 20). Phytoalexins are low mol wt antimicrobial compounds which plants can accumulate in quantities sufficient to inhibit the growth in culture of potential pathogens (6, 8, 11, 25, 28). It is thought that phytoalexin accumulation may be a mechanism by which plants resist the majority of microbes which they encounter (1, 12, 17).

Molecules that stimulate phytoalexin production have been isolated from various fungi. These molecules have been termed elicitors (18).

An elicitor of glyceollin accumulation in soybeans has been purified from the soybean fungal pathogen Pms³ (4). This Pms elicitor is a β -glucan and is a component of the mycelial wall (5).

Recently, an elicitor of glyceollin has been purified from commercial brewers' yeast extract. The purified elicitor is a glucan which appears to be structurally similar to the Pms elicitor (16). This suggests that soybeans can detect the presence of other fungi by recognition of a portion of their glucans. Such glucans are present in the cell walls of many different fungi (9).

The purpose of this work was to determine whether other higher plants have the ability to respond to Pms and yeast by recognition

of their glucan elicitors. Dark red kidney beans, *Phaseolus vulgaris*, and potatoes, *Solanum tuberosum*, have been chosen as models to test whether the Pms elicitor and the yeast elicitor will stimulate the accumulation of phytoalexins in other higher plants.

Dark red kidney beans respond to invasion by a variety of microorganisms including *Colletotrichum lindemuthianum*, the causal agent of anthracnose in beans, by turning brown at the site of inoculation and by accumulating a number of phytoalexins including phaseollin, phaseollidin, phaseollin isoflavin, and Kievitone (6-8, 11). This response is also stimulated by exposing the cut surfaces of kidney bean plants to as little as 100 ng of a polysaccharide elicitor which was isolated from the mycelia walls of *C. lindemuthianum* by Anderson-Prouty and Albersheim (3).

Potatoes respond to inoculation with a variety of microorganisms by accumulating a number of phytoalexins including rishitin, lubimin, and phytuberin (21, 22, 25, 28). A polysaccharide has been isolated from the mycelial walls of *Phytophthora infestans*, the causal organism of potato blight. Exposure of potatoes to this polysaccharide results in the accumulation of phytoalexins (10).

MATERIALS AND METHODS

An authentic sample of phaseollin was the generous gift of J. A. Bailey. An authentic sample of rishitin was kindly donated by G. D. Lyon.

The Pms glucan elicitor was obtained from B. Valent of this laboratory and was the fraction I elicitor purified by the procedure of Ayers *et al.* (4). The yeast elicitor was obtained from M. Hahn and was purified from yeast extract by the procedure of Hahn and Albersheim (16). A crude *C. lindemuthianum* elicitor, the concentrated water extract of autoclaved mycelial walls, was prepared by the procedure of Anderson-Prouty and Albersheim (3). Elicitor carbohydrate was quantitated by the anthrone method (13) using glucose as a standard and is reported as μ g of glucose equivalents.

Dark red kidney beans var. Charlevoix were obtained from the Idaho Seed Bean Co., Twin Falls, Idaho. The beans were soaked in sodium hypochlorite (0.75%) for 5 min, were then rinsed with distilled H₂O, and were planted in Vermiculite. Bean plants were grown under a photoregime of 14 hr of 2,500 ft-c illumination at 20 C and 10 hr of darkness at 18.5 C. Idaho russet potatoes were obtained from a local grocery and were surface-sterilized for 22 min with 80% ethanol before use.

Brewers' yeast was obtained from a local brewer's supply store and was washed with 10 mM potassium phosphate (pH 7.2) prior to use. The Pms was grown in still culture and was prepared for inoculation as previously described (4). Race α of *C. lindemuthianum* was maintained on agar slants containing the medium described by Mathur *et al.* (23). Spores of *C. lindemuthianum* were prepared for inoculation by adding 2 ml of sterile H₂O to an agar slant of the culture and by scraping spores from the surface of the agar with a spatula.

Inoculation of Bean Tissue and Extraction of Phytoalexins. Red

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³ Abbreviation: Pms: *Phytophthora megasperma* var. *sojiae*.

kidney bean cotyledons were excised from 6-day-old seedlings, were soaked in sodium hypochlorite (0.75%) for 5 min, and then rinsed with distilled H₂O. A thin epidermal strip was cut from the convex side of each cotyledon with the aid of a razor blade. Cotyledons were placed on moist filter paper in sterile Petri plates with their cut surfaces up. Solutions to be tested for elicitor activity were made 10 mM with respect to potassium phosphate (pH 7.2). Test solutions (50 μ l) were applied to each cotyledon. Sterile phosphate buffer was applied to cotyledons as a control. The cotyledons were incubated in the dark at 24 C for 22 to 24 hr.

Phytoalexins were extracted by soaking the treated cotyledons in 95% ethanol (2.5 ml/cotyledon) for 1 hr with swirling every 10 min. The ethanol extract was decanted and the cotyledons were reextracted with another volume of ethanol for 30 min. The ethanol extracts were combined and centrifuged at 12,000g for 10 min. The supernatant solution was evaporated to dryness under reduced pressure at 38 C and the residue was extracted with ethyl acetate (1 ml for each cotyledon originally extracted) for 1 hr. The ethyl acetate extract was blown dry under a stream of N₂ at room temperature. The residue was redissolved in ethyl acetate (10 μ l for each cotyledon originally extracted). This fraction is called the "concentrated phytoalexin extract."

Detection of Phytoalexins in Kidney Bean Cotyledon Extracts. Portions of the concentrated phytoalexin extracts were spotted onto 0.25-mm thin layer silica plates and the plates were developed with HCCl₃-methyl alcohol (100:4, v/v). The plates were dried and the presence of phytoalexins was detected by the use of a bioassay as previously described (3). In this bioassay, spores of *Cladosporium cucumerinum* are mixed with potato dextrose agar and the mixture is sprayed uniformly onto the thin layer plate. The plate is placed in a covered box and is incubated at 100% RH at 24 C for 48 to 72 hr. The spores of this fungus are dark. Regions where phytoalexins are present lack fungal growth and appear as white spots on a dark background.

Inoculation of Potatoes and Extraction of Rishitin. Ten cavities, each 2 cm deep \times 1.1 cm in diameter, were formed in each potato with a No. 6 cork borer. Solutions to be tested were supplemented with 500 μ g/ml penicillin. One ml of a test solution was pipetted into each cavity. The inoculated potatoes were covered with plastic wrap and were incubated at 19 C and 100% RH. After 48 hr, the tissue surrounding the treated cavities was removed with a No. 8 cork borer, diameter 1.8 cm, to a depth of 2.4 cm. Rishitin was extracted from the excised tissue by homogenizing the tissue in 70% ethanol (2 ml for every cavity) at 4 C in a Sorvall Omni-Mixer for 5 min. The homogenized tissue was filtered through cheesecloth and the liquid which passed through the cheesecloth was centrifuged at 16,000g for 20 min. The supernatant solution was evaporated to dryness under reduced pressure at 30 C. The residue was shaken with a mixture of H₂O and ethyl ether (40:60, v/v), 10 ml/original cavity. The ether layer was collected and the aqueous phase was reextracted two more times with ether. The ether extracts were combined and evaporated to dryness under reduced pressure at 30 C. The residue was redissolved in methanol (10 μ l for each cavity assayed). This fraction is called the "concentrated rishitin extract."

Identification and Quantitation of Rishitin. Portions of the concentrated rishitin extract were spotted on a 0.25-mm thin layer silica plate. The thin layer plate was developed with ethyl ether. Rishitin was visualized on the TLC plate as a pink spot when the plates were sprayed with a saturated solution of antimony trichloride in chloroform followed by heating at 60 C for 3 min. Rishitin was also detected as a fungistatic spot on plates which were bioassayed with *C. cucumerinum*. Rishitin was identified on thin layer plates by comparison of its migration to the migration of a rishitin standard.

Rishitin in the potato extracts was quantitated by a modification of the method of Sato and Tomiyama (26) as follows. The silica was scraped from areas corresponding to a rishitin standard from

thin layer plates on which potato extracts had been chromatographed. The silica was extracted with 20 ml of ethyl ether. The ether extract was evaporated to dryness under a stream of N₂ and the residue was dissolved in 1 ml of hexane. One ml of concentrated H₂SO₄ was added to the rishitin-containing hexane solution followed by vigorous mixing on a Vari-Whirl mixer. The *A* of the H₂SO₄ layer was measured at 500 nm and the amount of rishitin was calculated by comparison to a standard curve obtained using authentic rishitin. The identity of rishitin in the hexane solution was confirmed by gas chromatography and MS on a Hewlett-Packard model 5980A GC/MS using a glass column (2 mm \times 1.83 m) containing 1% O.V. 17 on Gas-chrom Q.

RESULTS

Response of Red Kidney Bean Cotyledons to Inoculation with Either Live Pms Mycelia or Yeast Cells. Cut red kidney bean cotyledons were inoculated with a suspension of 10⁷ yeast cells/ml or with a suspension of Pms mycelial fragments (100 mg/ml) as described. After 24 hr, the treated cotyledons appeared dark brown on the inoculated surfaces. Browning has previously been shown to be coupled with phytoalexin accumulation during a hypersensitive response of kidney bean hypocotyls to inoculation with an incompatible race of *C. lindemuthianum* (6, 8, 27). Phytoalexins were extracted from treated cotyledons as described. The concentrated phytoalexin extracts were chromatographed on thin layer silica plates and the plates were subjected to the *C. cucumerinum* bioassay (Fig. 1B). At least five fungistatic compounds are apparent and are, therefore, present in substantial quantities in extracts of the treated tissue. These compounds migrate with R_f values of 0.075, 0.15, 0.27, 0.36, 0.50 (Fig. 1B). The compound with the highest mobility in this chromatographic system migrates with the same R_f as a phaseollin standard.

Response of Kidney Bean Cotyledons to Pms and Yeast Elicitors. Cotyledons were inoculated with various concentrations of Pms elicitor and were incubated for 22 hr. Elicitor-treated tissue showed browning on the cut surface while control tissue exhibited no browning or only slight discoloration. As little as 1 μ g/ml of Pms elicitor was able to cause significant browning of cotyledons compared to control tissue. The phytoalexin extracts from Pms elicitor-treated tissue contain the five fungistatic compounds (Fig. 1A) that were present in extracts of cotyledons which were treated with live Pms (Fig. 1B). Only the fungistatic compound which migrates with an R_f of 0.27 is detectable in extracts of buffer-treated cotyledons. The five fungistatic compounds appear to accumulate in amounts which are approximately proportional to the concentration of Pms elicitor used. The most dramatic increase in concentration occurs with the compound which migrates with an R_f of 0.075.

Cotyledons were treated with spores of *C. lindemuthianum* and with a crude *C. lindemuthianum* elicitor preparation as a positive control. Both treatments resulted in intense browning of the cotyledons and in accumulation of large amounts of the five fungistatic compounds (Fig. 1A).

The application of yeast elicitor to red kidney bean cotyledons also resulted in tissue browning and phytoalexin accumulation; however, yeast elicitor was not as effective on a weight basis as Pms elicitor in stimulating this response. Solutions of yeast elicitor containing 2, 25, and 100 μ g of glucose equivalents/ml were applied to cut cotyledons. Significant browning was observed with the 25 μ g/ml and the 100 μ g/ml treated tissue but not with the 2 μ g/ml treated tissue. Significant quantities of the five fungitoxic compounds were observed only in extracts of the cotyledons which had been treated with 100 μ g/ml of the yeast elicitor.

Accumulation of Rishitin in Potatoes in Response to Inoculation with Live Pms and with Pms and Yeast Elicitors. Potatoes were treated with live Pms and with various concentrations of both Pms and yeast elicitors. After 48 hr, the tissue surrounding the treated

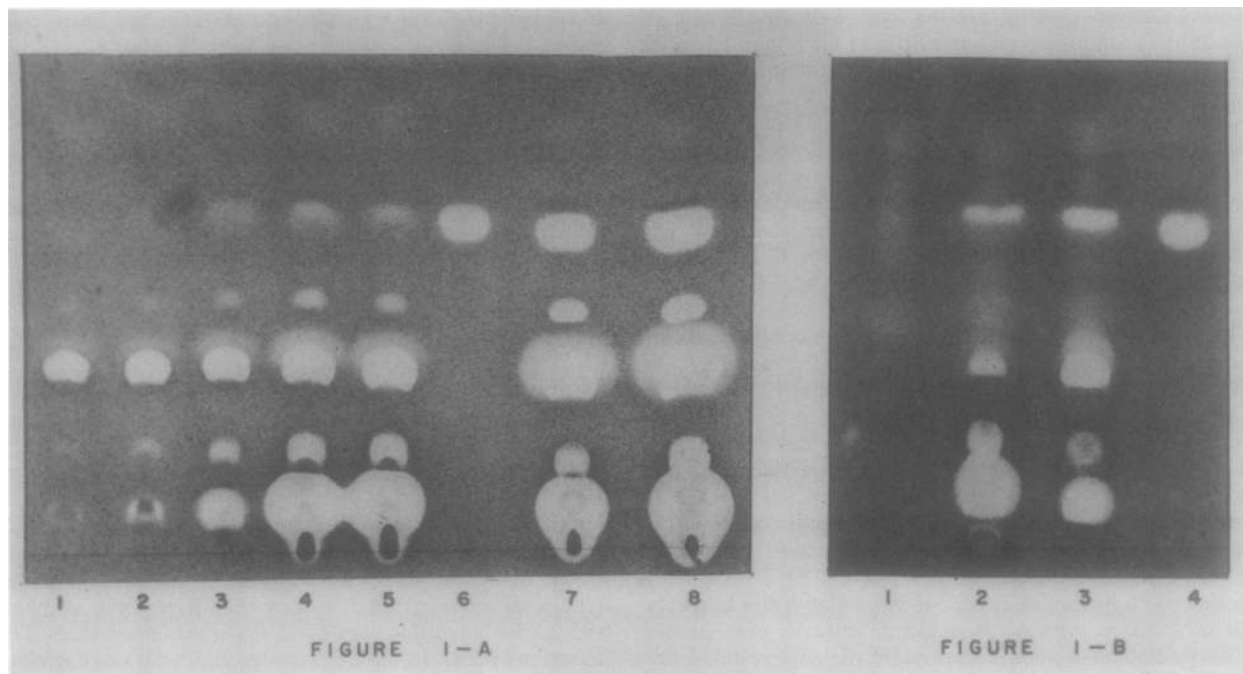


FIG. 1. Occurrence of fungistatic compounds in extracts of red kidney bean cotyledons. Cut surfaces of cotyledons were inoculated with solutions to be tested ($50 \mu\text{l}$ /cotyledon). Cotyledons were then incubated in the dark at 24°C for 22 to 24 hr. A: 30 cotyledons were extracted as described under "Materials and Methods." Portions ($20 \mu\text{l}$) of the concentrated phytoalexin extracts were spotted on thin layer silica plates. The thin layer plates were developed with chloroform and methanol (100:4, v/v) and were subsequently bioassayed with *C. cucumerinum*. 1: control tissue treated with sterile buffer; 2: tissue treated with $1 \mu\text{g}$ of Pms elicitor; 3: tissue treated with $5 \mu\text{g}/\text{ml}$ Pms elicitor; 4: tissue treated with $25 \mu\text{g}/\text{ml}$ Pms elicitor; 5: tissue treated with $125 \mu\text{g}/\text{ml}$ Pms elicitor; 6: phaseollin standard; 7: tissue treated with $25 \mu\text{g}/\text{ml}$ crude elicitor from *C. lindemuthianum*; 8: tissue treated with spores of *C. lindemuthianum* (approximately $4 \times 10^6/\text{ml}$). B: 20 cotyledons were extracted as described. Portions of the concentrated phytoalexin extracts ($25 \mu\text{l}$) were chromatographed on thin layer plates. The dried plates were bioassayed with *C. cucumerinum*. 1: control tissue treated with sterile buffer; 2: tissue treated with Pms mycelial fragments ($0.1 \text{ g}/\text{ml}$); 3: tissue treated with yeast cells ($10^7/\text{ml}$); 4: phaseollin standard.

areas was removed, weighed, and rishitin extracted and quantitated as described. Treatment of potatoes with live Pms resulted in the accumulation of $28 \mu\text{g}$ of rishitin/g fresh weight tissue while control tissue contained only $0.95 \mu\text{g}$ of rishitin/g fresh weight.

The Pms and yeast elicitors were both very effective in stimulating potatoes to accumulate rishitin. Significant quantities of rishitin over control tissue resulted from the treatment of potatoes with as little as $0.16 \mu\text{g}/\text{ml}$ Pms elicitor and $0.1 \mu\text{g}/\text{ml}$ yeast elicitor. The amount of rishitin extracted is plotted versus the concentration of elicitor used in the treatment (Fig. 2). The Pms elicitor stimulates tissue to accumulate a maximum of $29 \mu\text{g}$ of rishitin/g fresh weight and the yeast elicitor stimulates a maximum accumulation of $20.0 \mu\text{g}/\text{g}$. The concentration of elicitor which resulted in one-half of the maximum accumulation of rishitin was $0.60 \mu\text{g}/\text{ml}$ for the Pms elicitor and $0.37 \mu\text{g}/\text{ml}$ for the yeast elicitor (Fig. 2 inset).

DISCUSSION

Red kidney bean cotyledons respond to inoculation with Pms mycelia, with yeast cells, and with spores of the bean pathogen, *C. lindemuthianum*, by turning brown at the site of inoculation and by accumulating at least five fungistatic compounds (Fig. 1). This response in red kidney bean cotyledons is also stimulated by exposing cut cotyledons to purified glucans from Pms and from yeast (Fig. 1A). The Pms and yeast glucans are known to be potent elicitors of glyceollin accumulation in soybeans.

The five fungistatic compounds which accumulate in treated kidney bean cotyledons migrate with R_f values of 0.075, 0.15, 0.27, 0.36, and 0.50 on thin layer silica plates developed with HCCl_3 -methyl alcohol (100:4, v/v). A phaseollin standard migrates with an R_f of 0.50 in this chromatographic system.

Previous studies have shown that kidney bean hypocotyls re-

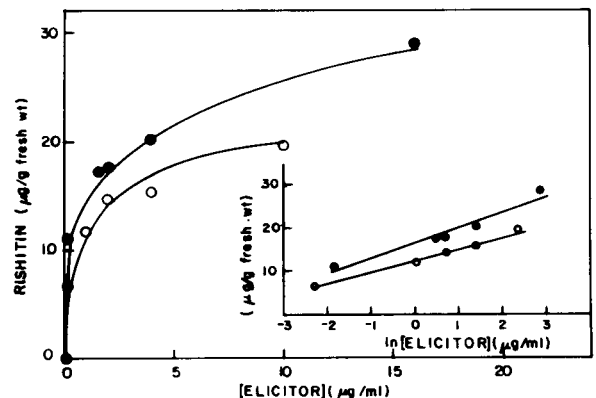


FIG. 2. Accumulation of rishitin in potato tubers following exposure to glucan elicitors. Tissue surrounding 10 inoculated cavities was extracted and rishitin was determined. Micrograms of rishitin/g fresh weight of potato tissue extracted are plotted versus the concentration of elicitor applied. Inset: concentration of elicitor which resulted in one-half of the maximum accumulation of rishitin was calculated from a linear regression of the μg of rishitin/g fresh weight versus the logarithm of elicitor concentration. (●): Pms elicitor; (○): yeast elicitor.

spond to inoculation with an incompatible race of *C. lindemuthianum* by accumulating the phytoalexins, kievitone, phaseollin isoflavin, phaseollidin, and phaseollin (6). These phytoalexins have reported R_f values of 0.075, 0.27, 0.37, and 0.50, respectively, on thin layer silica plates developed with HCCl_3 -ethyl alcohol (100:3, v/v) (7). These results suggest that four of the fungistatic compounds which accumulate in red kidney bean cotyledons following exposure of the cotyledons to the glucan elicitors from Pms or yeast are the phytoalexins which have previously been

reported to accumulate in kidney bean hypocotyls following inoculation with *C. lindemuthianum*.

Potatoes accumulate rishitin following exposure to either living Pms mycelial fragments (Fig. 2) or to the glucans purified from Pms and from yeast extract (Fig. 2). Potatoes accumulated 28 μg of rishitin/g fresh weight following inoculation with live Pms and accumulated 29 μg of rishitin/g fresh weight following exposure to Pms glucan. Therefore, the Pms glucan is as effective in this assay as live Pms in eliciting the accumulation of rishitin in potatoes. The yeast glucan is also a very potent elicitor of rishitin accumulation. Potatoes accumulated 19.5 μg of rishitin/g fresh weight following exposure to the yeast glucan.

Recently, Lisker and Kuć (21) reported that the Pms glucan (20 μg /potato slice) elicited the accumulation of low levels of rishitin and lubimin. The amounts of these phytoalexins which accumulated in their experiments in response to the Pms glucan were much lower than the amounts accumulated in response to various live and heat-killed fungi. There are significant experimental differences between the biological assays conducted by Lisker and Kuć and the biological assays described in this paper. Such differences may account for the discrepancies in these two studies in the amount of rishitin accumulated in potatoes following exposure to Pms glucan.

The original discovery that a β -glucan from the mycelial walls of Pms could stimulate soybeans to accumulate the phytoalexin glyceollin indicated that soybeans were capable of detecting and responding to the presence of a fungus by recognizing a molecule synthesized by that fungus. The importance of glucans in this recognition process was supported by the characterization of an elicitor of glyceollin accumulation which was purified from commercial brewers' yeast extract. The purified yeast elicitor is a polysaccharide which consists largely, if not entirely, of glucosyl residues (16).

The results presented in this paper demonstrate that the ability to detect and respond to fungi by recognition of fungal glucans is not unique to soybean. Potato and red kidney bean as well as soybean respond to the highly purified Pms glucan by accumulating phytoalexins. These plants appear to respond to Pms following recognition of the same mycelial wall component. The stimulation of phytoalexin accumulation in soybean, potato, and red kidney bean by the structurally similar yeast glucan supports the hypothesis that plants may respond to a variety of fungi by recognizing the same or similar mycelial wall components. These conclusions are supported by other work. For example, the Pms glucan stimulates suspension cultures of sycamore cells and parsley cells to produce increased amounts of phenylalanine ammonia-lyase activity (15). This enzyme is thought to participate in the biosynthesis of phytoalexins in many plants (14, 15, 24). Molecules which elicit phytoalexin accumulation in kidney beans have been isolated from three species of *Colletotrichum*. The elicitors from the three *Colletotrichum* species are glucose-containing polysaccharides (2, 3).

Many plants have been shown to respond to a large variety of fungi by accumulating phytoalexins. An efficient means by which a plant could detect diverse fungi would be the recognition of molecules which are common to these fungi. Beta-linked glucans with compositions similar to the elicitors isolated from Pms and from brewers' yeast are cell wall components of many fungi (9). It may be that these glucans are important determinants in the defense response of plants to invasion by these fungi.

stages of the experimentation and of M. McNeil who performed the combined gas chromatography-mass spectrometry of rishitin.

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