Studies on the Mode of Action of the Phytoalexin Phaseollin

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ABSTRACT

Phaseollin at 15 μg/ml inhibited dry weight increase of *Rhizoctonia solani* for 12 to 18 hr; 47 μg/ml caused an initial decrease in fungal dry weight. Phaseollin decreased the ability of *R. solani* to take up glucose-U-^{14}C. Mycelium which had previously incorporated ^{14}C rapidly released materials containing ^{14}C upon exposure to phaseollin. The respiration rate of actively growing mycelia was decreased by phaseollin, but the respiration rate of "starved" mycelia was stimulated. When *R. solani* was exposed to phaseollin-^{14}C for 1 hr and then fractionated, most of the ^{14}C was associated with the hyphal fraction removed by centrifugation at 500 *g* for 15 min. Exposure of *R. solani* hyphae to phaseollin resulted in an immediate cessation of protoplasmic streaming and in shrinkage of the protoplast from the hyphal tip. Phaseollin at 23 μg/ml and above rapidly lysed erythrocytes. There was little effect of phaseollin on endogenous or exogenous respiration rate of bean hypocotyl tissue, but phaseollin caused a rapid release of $^{86}Rb^{+}$ from hypocotyl tissue into which $^{86}Rb^{+}$ had previously been incorporated. The hypothesis is advanced that phaseollin acts on the plasma membrane or affects some process needed for membrane function.

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Phaseollin is a phenolic pterocarpan derivative (27). Since it is produced by bean (*Phaseolus vulgaris* L.) in response to fungal infection and possesses antibiotic activity, it has been classified as a phytoalexin (4, 26). Some evidence suggests that phaseollin may function in an active induced-resistance mechanism to fungal invasion of bean (3, 5, 29); however, some investigators have suggested that this compound may be of secondary importance in certain host-parasite interactions (30). The evidence that phaseollin plays a role in disease resistance has been indirect.

More direct evidence for in situ action or lack of it might be obtained in at least two ways through an in vitro study of its mode of action against fungi. Firstly, when phaseollin is operative in disease resistance, then the development of an invading fungus should be affected by its antibiotic action. If the effects of phaseollin in vitro were known, then a similar response might be observed in situ, provided this compound is functional in disease resistance. Secondly, if the mechanism through which phaseollin inhibits fungal growth could be elucidated, it might be possible specifically to annul its antibiotic action in situ without altering the other induced changes associated with host-parasite interaction. If specific annulment of phaseollin's action should render the bean plant more susceptible to attack by fungi, this would provide conclusive evidence that this compound plays a direct role in disease resistance.

This study was therefore undertaken in an attempt to elucidate the mode of action of the phytoalexin phaseollin. *Rhizoctonia solani* Kuehn (our isolate RB), a pathogen of bean, is sensitive to phaseollin (29), and was employed as the main test organism.

MATERIALS AND METHODS.—*Rhizoctonia solani* was grown in 25-ml Erlenmeyer flasks containing 4.0 ml of a filter-sterilized liquid medium which contained per liter: 20 g glucose; 3.0 g Bacto asparagine (Difco Laboratories, Detroit, Mich.); 0.87 g K_2HPO_4; 6.12 g KH_2PO_4; 0.14 g $MgSO_4 \cdot 7 H_2O$; and 1.0 ml Steinberg's (35) microelement solution. Cultures were incubated at 30 C on a reciprocal shaker operated at 200 strokes (3.8 cm)/min. Inoculum consisted of mycelial discs (4 mm) of *R. solani* obtained from colonies growing on bacterial filters (Triacetate Metricel, 0.2-μ pore size, Gelman Instrument Company, Ann Arbor, Mich.) which had been placed on the surface of potato-dextrose agar (PDA). The pads were cut from the colonies with a cork-borer about 1 cm from the margin of the colonies. They were washed in H_2O, and four discs (1.5 ± 0.4 mg dry wt) were used to inoculate each flask of medium. After 24 hr of growth, spherical pads of mycelium surrounded the initial flat mycelial discs. They weighed about 6.5 ± 0.6 mg (dry wt)/flask. Growth curves for *R. solani* in this system revealed that at 24 hr of incubation, the cultures had entered the "exponential phase" of growth. Except for the studies dealing with microscopic observations of the fungus after exposure to phaseollin, these 24-hr-old mycelial pads were used to study the effect of phaseollin on *R. solani*.

Dry weights of the fungus were determined by collecting and washing the mycelium on tared filter paper (Whatman No. 50), using a vacuum filtration apparatus and weighing after a 24-hr exposure to 75 C.

Bean (*Phaseolus vulgaris* L. 'Red Kidney') seedlings were grown as previously reported (37). One- to 2-mm-thick cross sections of hypocotyls from 7- to 10-day-old bean plants were used for respiration and $^{86}Rb^{+}$ leakage studies.

Crystalline phaseollin was prepared by the procedure of VanEtten & Bateman (36). Quantitation of phaseollin was accomplished by using its reported molar extinction coefficient ($\log \epsilon = 3.96$) in ethanol at 279 nm (4).

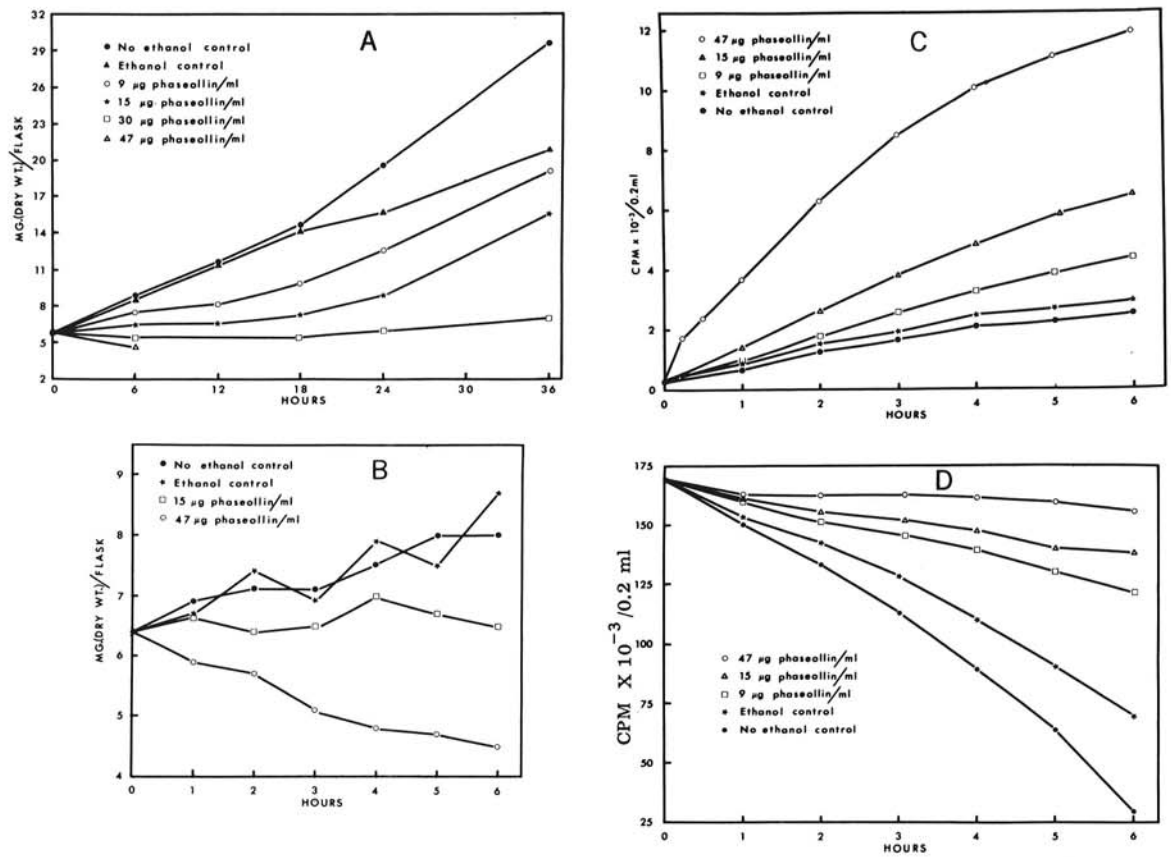

Fig. 1.A,B) Growth of *Rhizoctonia solani* in presence of phaseollin. **A)** Growth response over a 36-hr interval. **B)** Response over a 6-hr interval. Each data point represents the mean of four replicates. **C)** Loss of radioactivity from *R. solani* previously grown on glucose-U-^{14}C induced by exposure to phaseollin. **D)** Removal of D-glucose-U-^{14}C from medium by *R. solani* in presence of phaseollin. At the indicated intervals, 0.2-ml samples of the medium were withdrawn from the reaction vessels and assayed for ^{14}C. The data points represent the average of two samples.

Initial studies indicated that phaseollin was inhibitory to *R. solani* only when in solution. A number of organic solvents were effective, but at the minimal concentration (0.5%) needed to maintain phaseollin in solution, all those organic solvents tested inhibited *R. solani* in the absence of phaseollin. Ethanol and dimethylsulfoxide (DMSO) inhibited least, and one or the other of these was used as the solvent in all experiments. The appropriate amount of phaseollin in solvent was added to media to give a final solvent concentration of 0.5%.

^{14}C-labeled phaseollin was prepared by using acetate-U-^{14}C as a precursor to phaseollin (12). Bean hypocotyls (18 g fresh wt) bearing newly formed lesions caused by *R. solani* were collected, and the pith area was removed with a cylindrical glass tube (ca. 1.0 mm outside diam). The basal ends of the hypocotyls were sealed with petroleum jelly, and the central cavity thus formed was filled with a solution containing sodium acetate-U-^{14}C (28 μc/μmole, Calatomic, Los Angeles, Calif.). A total of 1 mc of acetate-U-^{14}C was added by making additions of the acetate solution (0.5 mc/ml) to the hypocotyl cavities at 12-hr intervals over a period of 2 days. After 2 days of further incubation, phaseollin was extracted from the hypocotyls using the procedure of VanEtten & Bateman (36). The final extract was chromatographed on silica gel (250 μ thick) thin-layer chromatography (TLC) plates with pentane:diethyl ether: acetic acid (75:25:1) as the developing solvent (11, 36). The area containing phaseollin on the TLC plates was eluted with 95% ethanol. Unlabeled phaseollin was added to the ethanol solution, and ^{14}C phaseollin was crystallized by the slow addition of H_2O. The crystalline phaseollin had an activity of 2.72 x 10^3 dpm/μg.

All ^{14}C samples were assayed in 15 ml of the toluene:dioxane:methanol scintillation solution of Lips & Beevers (23) with a Packard Tri-Carb liquid scintillation spectrometer, Model 3002 or 3375. Where the amount of quench varied between samples within a given experiment, the counts per minute values observed were converted to disintegrations per minute by the external standard method of quench correction. Radioactivity is reported as counts per minute where the amount of quench was constant within an experiment.

$^{86}Rb^+$ was assayed by drying liquid samples on planchets at 75 C overnight prior to counting with a Nuclear-Chicago (Model 447) gas flow counter. Activ-

ity is expressed as counts per minute.

All experiments were repeated at least once, and a representative result of each experiment is presented in the text.

RESULTS.—*Growth responses of R. solani in the presence of phaseollin.*—The growth rate of *R. solani* was comparable in the presence and absence of 0.5% ethanol during the first 18 hr of incubation. However, the growth rate in the presence of ethanol always decreased between 18 and 24 hr (Fig. 1-A). Since ethanol was used in most experiments as the solvent for phaseollin, experimental periods were thus generally limited to less than 18 hr. In addition, a control containing 0.5% ethanol (hereafter referred to as the ethanol control) and a control without ethanol (no ethanol control) were used in all studies to distinguish possible effects of ethanol.

After the addition of phaseollin to a concentration of 9 μg/ml (2.8×10^{-5} *M*) of medium, fungal growth continued, but at a lower rate than in the controls (Fig. 1-A). At a concentration of 15 μg/ml (4.7×10^{-5} *M*), there was essentially no growth for the first 12 to 18 hr; then growth resumed. Phaseollin concentrations of 30 and 47 μg/ml (9.3×10^{-5} and 1.46×10^{-4} *M*, respectively) resulted in a decrease in mycelial dry weight initially. This effect was observed within 1 hr after exposure to 47 μg/ml (Fig. 1-B). The mycelial pads had lost ca. 25% of their dry wt after 6 hr. One experiment was carried out in which the mycelial pads (original dry wt ca. 6.1 mg) were autoclaved for 25 min. Such treatment should destroy the fungal membranes and permit leakage of cellular constituents from the cells. The dry weight of these mycelial pads averaged 3.7 mg. When nonautoclaved pads were treated with 47 μg/ml of phaseollin for 6 hr, the average dry weight decreased to 4.3 mg, indicating that phaseollin caused the fungus to lose a large portion of its internal constituents. Despite the loss in dry weight on exposure to phaseollin, live fungal tissue was still present, because after another 36 to 48 hr of incubation the fungus began to grow. In another experiment, *R. solani* pads were exposed to fresh solutions of phaseollin (47 μg/ml) every hour for four treatments. Upon incubation for an additional 36 to 48 hr after the fourth treatment, new growth was visible. Phaseollin thus appears to be fungistatic rather than fungicidal under these conditions.

Effect of phaseollin on leakage of metabolites and glucose uptake by R. solani.—The internal metabolites of *R. solani* were labeled with ^{14}C by growing *R. solani* on the standard growth medium supplemented with 1 μc/ml of glucose-U-^{14}C (2.8 μc/μmole, Calatomic). After 24 hr of growth, the ^{14}C-labeled mycelial pads (48) were washed with three 75-ml portions of unlabeled growth medium. After the third wash, the ^{14}C-labeled mycelial pads were transferred to fresh medium (four pads/4.0 ml) in 25-ml Erlenmeyer flasks. After various treatments, 0.2 ml-aliquots of growth medium were withdrawn at designated time intervals. The amount of ^{14}C present in each aliquot was interpreted as a measure of the metabolites released from the fungal cells.

Phaseollin-treated mycelium released ^{14}C-substances into the growth medium at a faster rate than the nontreated mycelium (Fig. 1-C). This response was observed at all phaseollin concentrations tested (9, 15, and 47 μg/ml), and was especially rapid in the presence of 47 μg phaseollin/ml.

To test the effect of phaseollin on rate of glucose uptake by *R. solani*, the fungus was grown for 24 hr in the usual manner, then transferred to the standard growth medium containing 0.5 μc/ml of D-glucose-U-^{14}C (10 μc/μmole, Calatomic) and one-twentieth the normal amount of glucose and asparagine. The rate of glucose-U-^{14}C uptake was measured by determining the amount of ^{14}C present in 0.2-ml aliquots of the growth medium withdrawn at various time intervals.

Phaseollin at all concentrations tested inhibited the removal of glucose-U-^{14}C from the culture medium by *R. solani* (Fig. 1-D). Almost complete inhibition was observed at the highest phaseollin concentration (47 μg/ml).

Effects of phaseollin on exogenous and endogenous respiration of R. solani.—Oxygen uptake by *R. solani* was measured with a Gilson differential respirometer. All studies were made at 30 C, and the respirometer flasks were shaken at 150 strokes/min. The center wells of the respirometer flasks contained 0.2 ml of 10% KOH, and in all experiments the respirometer flasks and contents were equilibrated for 1 hr before the addition of phaseollin in its carrier solvent. The effect of phaseollin on "exogenous" O_2 uptake was studied by adding four 24-hr-old pads of *R. solani* to 4.0 ml of the standard growth medium contained in respirometer flasks. For endogenous respiratory measurements, 24-hr-old *R. solani* pads were "starved" by incubating them in the standard growth medium minus glucose and asparagine for 24 hr; the measurements were made in respirometer flasks containing four *R. solani* pads and 4.0 ml of the standard growth medium minus glucose and asparagine. In order to minimize the chance that the phaseollin solvent would be utilized as a carbon source by *R. solani*, DMSO was used as the carrier solvent for phaseollin for the studies of endogenous respiration.

The effect of phaseollin on O_2 uptake by *R. solani* depended on the nutritional state of the mycelium. When *R. solani* was actively growing in the presence of an external carbon source, O_2 uptake was inhibited by the addition of phaseollin (Fig. 2-A). With increasing concentrations of phaseollin there was increasing inhibition. The rate of O_2 uptake in the treatments containing 47 μg/ml of phaseollin was only ca. 40% of the controls.

The rate of O_2 uptake by 24-hr starved mycelium was ca. 0.25 the rate of a similar amount of actively growing mycelium (Fig. 2-A, B). However, when the starved mycelium was exposed to phaseollin, there was a marked stimulation of O_2 uptake (Fig. 2-B). The respiration rate of the starved mycelium after exposure to 9, 15, or 47 μg/ml of phaseollin was almost double that of the controls.

Uptake of phaseollin by R. solani.— Four 24-hr-old pads of autoclaved or nonautoclaved *R. solani*

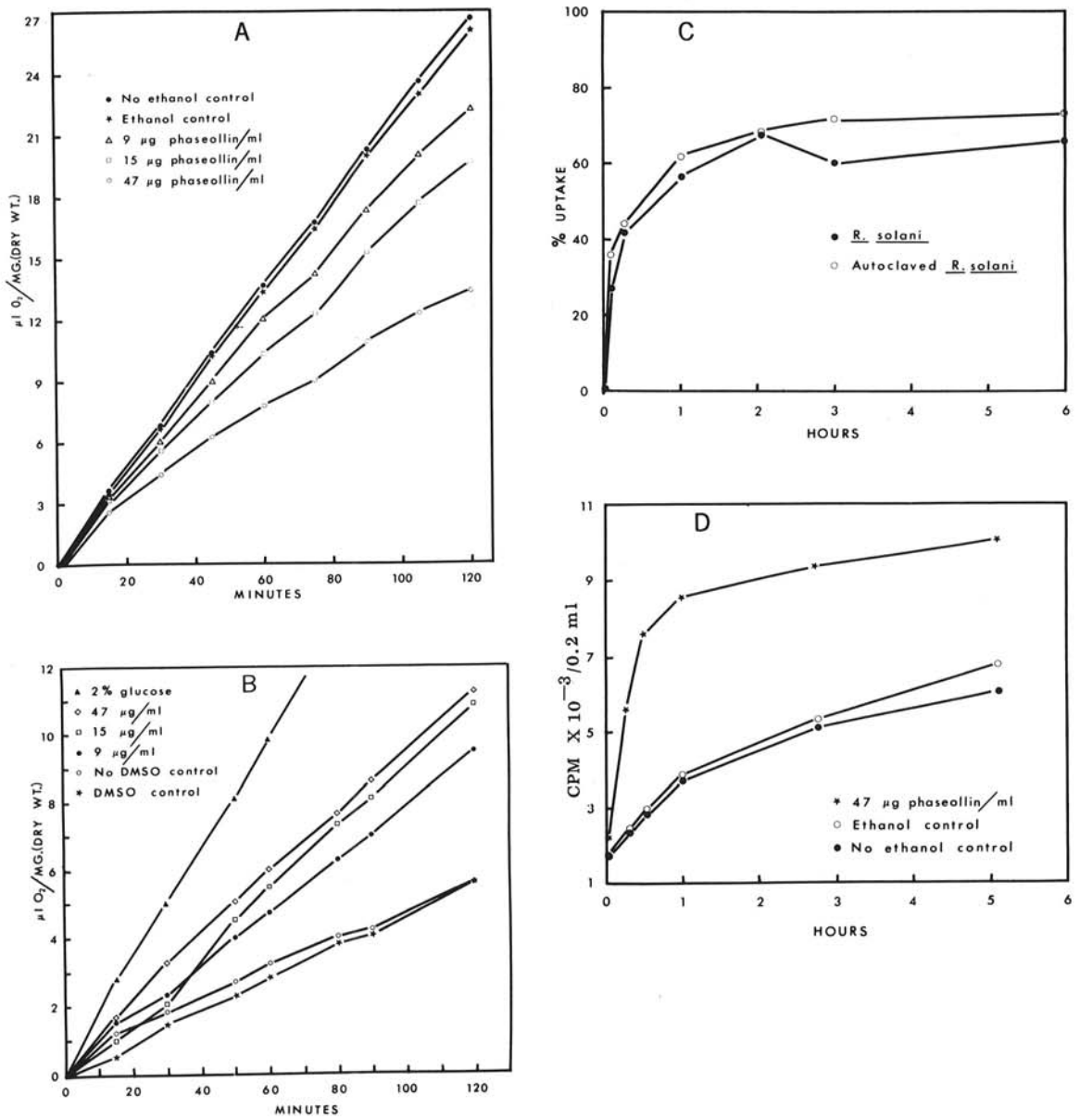

Fig. 2. Effect of phaseollin on **A)** the "exogenous" respiration rate of *Rhizoctonia solani*; and **B)** the endogenous repiration rate of *R. solani*. By measuring O_2 uptake of actively growing mycelium in the presence of 2% glucose and 0.3% asparagine, exogenous respiration was determined. The rate of O_2 uptake by mycelium that had been incubated in the absence of an added carbon source for 24 hr was considered as endogenous respiration. A final concentration of 0.5% dimethyl sulfoxide (DMSO) was used as the solvent for phaseollin in the endogenous respiration studies. Time 0 in the figures is 15 min after the additions were made. Each data point represents the mean of three replicates. **C)** Uptake of phaseollin from solution by "living" and autoclaved mycelium of *R. solani*. Phaseollin was added to the flasks to give a concentration of 15 µg/ml of phaseollin. At the indicated intervals, samples of the medium were withdrawn and phaseollin extracted. After 6 hr, phaseollin was similarly extracted from flasks which had contained no mycelium. Phaseollin equal to 12.7 µg/ml of the original solution was recovered from these flasks, and this value was therefore used to compare per cent uptake in the reaction vessels containing mycelium. Each data point is an average of two samples. **D)** Effect of phaseollin on release of $^{86}Rb^+$ from bean hypocotyl tissue which had previously incorporated this cation. At the indicated intervals, samples of the solution bathing the hypototyl tissue were withdrawn and assayed for $^{86}Rb^+$. Each data point represents an average of two samples.

were added to each of a number of 25-ml Erlenmeyer flasks containing 4.0 ml of 0.05 *M* potassium phosphate buffer at pH 5.8. Phaseollin was then added to the flasks to give a concentration of 15 μg/ml. At time intervals, 2.0-ml samples of the buffered solution were removed from each flask and partitioned twice with four volumes of petroleum ether (85% hexane). The petroleum ether fraction (containing phaseollin) was dried, and the residue redissolved in 2.0 ml of 95% ethanol. Phaseollin was identified by its ultraviolet absorption spectrum and its concentration calculated from the absorbance at 279 nm.

The removal of phaseollin from solution by both autoclaved and nonautoclaved fungal pads was rapid for the first 10 to 15 min, then leveled off after 1 hr (Fig. 2-C). About 70% of the phaseollin added to the medium was taken up by both types of mycelia. Since autoclaved mycelium removed phaseollin from solution as rapidly, and to approximately the same extent, as nonautoclaved mycelium (Fig. 2-C), phaseollin removal from solution by *R. solani* was not an energy-requiring process.

Measure of degradation of phaseollin by R. solani.—Twenty-four-hr old pads of *R. solani* were exposed to 15 μg/ml of ^{14}C-phaseollin (2.72×10^3 dpm/μg; total of 1.63×10^5 dpm) in airtight 130-ml containers (four discs in 4.0 ml of 0.05 *M* potassium phosphate buffer, pH 5.8). Since phaseollin was removed from solution by autoclaved mycelium, autoclaved fungal pads were included in degradation studies as controls. The containers were shaken at 150 strokes/min at 30 C for 1 hr. The CO_2 evolved was trapped in Whatman No. 3 paper strips soaked with 10% KOH. After a 1-hr incubation period, 2.0 ml of the bathing buffer was withdrawn, placed in a scintillation vial, and dried. The KOH-soaked papers were added to 15 ml of scintillation solution. The *R. solani* pads were placed in a scintillation vial containing ca. 2.0 ml of 95% ethanol and homogenized in the vial with a VirTis 45 micro homogenizer. The homogenizer blades were rinsed with about 10 ml of ethanol which was added to the vial. The vial was centrifuged for 10 min at ca. 500 *g*, and the supernatant solution was transferred to another scintillation vial and taken to dryness. This extraction of the mycelial pads was repeated 3 more times. After the fourth extraction, 0.5 ml of NCS solubilizer (Amersham/Searle Company, Arlington Heights, Ill.) was added to the vial containing the ethanol-insoluble mycelial residue; then the vial was heated at ca. 50 C for 24 hr. After this treatment, 15.0 ml of scintillation solution was added to the digest.

The dried residues from the bathing buffer solution and dried residues from the ethanol extract of the mycelium were washed 4 times with 200-μliter batches of ethanol. These washes were streaked on silica gel TLC plates and chromatographed as described above. The area containing phaseollin on the chromatograms and other areas of the chromatograms were collected and added to scintillation vials. The ethanol-washed vials originally containing the residues were also counted. As many of the manipulations as possible were carried out in scintillation vials in an attempt to minimize the loss of ^{14}C and to account for total ^{14}C added.

In this study, ca. 90% of the ^{14}C added was accounted for by the analysis employed (Table 1). Of this 90%, ca. 7-8% was not subjected to chromatography; that is, it remained in the extracting vials, and in the ethanol-insoluble mycelial residue. Of the ^{14}C that was subjected to chromatography, 81 to 84% migrated as phaseollin. Because ca. 20% did not migrate as phaseollin, some degradation of the ^{14}C-labeled phaseollin was indicated. It is suggested that breakdown of phaseollin possibly occurred while the sample was applied to silica gel TLC plates. Degradation of phaseollin when left in a dry state on such plates has been observed previously (36). Because of the large volume of material applied to the plates, it was necessary to expose the extracts to such a

TABLE 1. Recovery of ^{14}C from *Rhizoctonia solani* cultures after exposure to ^{14}C-labeled phaseollin[a]

Source of ^{14}C	Disintegrations/minute	
	Nonautoclaved mycelium	Autoclaved mycelium
Chromatographed as phaseollin[b]	103,932	112,320
Did not chromatograph as phaseollin[b]	23,722	21,695
^{14}C that was not chromatographed[c]	13,179	9,573
"$^{14}CO_2$"[d]	21	23
Total	140,854	143,611
%^{14}C on chromatogram that migrated as phaseollin	81	84

[a] Reactions were carried out in 130-ml airtight vessels containing four 24-hr-old autoclaved or nonautoclaved *R. solani* pads and 4.0 ml of 0.05 *M* potassium phosphate buffer, pH 5.8, made to 15 μg/ml of phaseollin-^{14}C (2.72×10^3 dpm/μg, total dpm = 1.63×10^5) and 0.5% ethanol. Exposure time was 1 hr.

[b] The material extracted from the buffered bathing solution and the material extracted from the mycelium were pooled and streaked on thin-layer chromatography plates. After development, the areas of the chromatograms containing phaseollin and the remaining areas were collected and the ^{14}C present was determined.

[c] This fraction represents the ^{14}C that remained associated with the digest of the ethanol insoluble mycelial residue, and the ^{14}C remaining in the scintillation vials in which the extractions were performed.

[d] Strips of Whatman No. 3 filter paper were soaked in 10% KOH and held in containers above the reaction mixtures to trap the CO_2 evolved. At the end of the 1-hr incubation period, these papers were added to scintillation vials and counted.

condition for a short period of time. The fact that the same amount of breakdown occurred in the autoclaved control sample indicates that biological degradation by *R. solani* was not involved. From these results, it seemed likely that when ^{14}C-labeled phaseollin was added to *R. solani* pads, most of the ^{14}C would remain in the intact phaseollin molecule for at least 1 hr under the condition employed.

Binding of phaseollin to cellular components.—A total of twenty-four 24-hr-old *R. solani* pads (ca. 39 mg dry wt) were added to 24.0 ml of 0.05 *M* potassium phosphate buffer at pH 5.8 in a 125-ml Erlenmeyer flask. Phaseollin-^{14}C (972 dpm/μg) was added to give a concentration of 15 μg/ml. After incubating for 1 hr at 30 C (100 strokes/min), the pads were washed 5 min with 25 ml of a solution of 0.32 M sucrose and 0.5% ethanol in 0.05 *M* potassium phosphate buffer at pH 5.8. The fungal pads were then homogenized for 30 sec in 10.0 ml of the above sucrose-ethanol solution with a VirTis 45 micro homogenizer. This homogenate was transferred to a 75-ml Bronwill MSK flask containing ca. 12 ml of 0.25-0.30 mm glass beads. The flask was shaken for 45 sec at 4,000 cycles/min in a Bronwill MSK cell homogenizer. Microscopic examination of the homogenate revealed that most "cells" (regions between two septa) were broken by this treatment. An aliquot (5.0 ml) of the homogenate was withdrawn from the MSK flask and centrifuged at 500 *g* for 15 min. The pellet was transferred to a scintillation vial, and the supernatant fluid was centrifuged at 30,000 *g* for 1 hr. This pellet was transferred to a scintillation vial, and the supernatant fluid centrifuged at 178,000 *g* for 11 hr. This pellet and an aliquot of the supernatant fluid were transferred to separate scintillation vials for counting. All cell fragmentations and centrifugations were carried out between 4 and 10 C.

When *R. solani* mycelium exposed to phaseollin-^{14}C for 1 hr was fractionated, most of the radioactivity was associated with the 500 *g* pellet (Table 2). A considerable amount of phaseollin remained in the supernatant solution even after centrifuging at 178,000 *g* for 11 hr. In one experiment, autoclaved mycelium which had been exposed to phaseollin-^{14}C was fractionated. Upon fractionation, about 87% of the phaseollin taken up by this mycelium was recovered in the 500 *g* pellet; 2.8% was recovered in the 30,000 *g* pellet. When the 30,000 *g* supernatant solution was stored for 2 hr at 4 C, phaseollin crystallized from solution. Phaseollin never crystallized out of the 30,000 *g* or 178,000 *g* supernatant solution from nonautoclaved mycelium, even when held at 4 C for 24 hr. This suggests that a portion of the phaseollin in nonautoclaved mycelium preparations is "bound" to some soluble cellular constituent.

Phase microscopy of R. solani during exposure to phaseollin.—Portions of the advancing mycelial margin of *R. solani* were removed from the bacterial filter and placed on microscope slides in a few drops of the liquid growth medium containing 0.5% ethanol and covered with a cover slip. To examine the effect of phaseollin on *R. solani*, a few drops of growth medium containing phaseollin (47 μg/ml) were placed by the edge of the cover slip, then drawn between the cover slip and slide by placing absorbent paper on the opposite side of the cover slip. The behavior of the mycelium and its contents in the presence or absence of phaseollin was observed with a Carl Zeiss phase contrast microscope.

TABLE 2. Binding of phaseollin-^{14}C by cellular fractions of *Rhizoctonia solani*[a]

Fractions	Disintegrations/minute	%[c]
500 *g* pellet	56,802	73.5
30,000 *g* pellet	4,394	5.7
178,000 *g* pellet[b]	532	0.7
178,000 *g* supernatant solution	15,550	20.1

[a] *Rhizoctonia solani* was incubated for 1 hr in the presence of 15 μg/ml of ^{14}C-labeled phaseollin (970 dpm/μg). After several washes, the hyphae were fragmented with a VirTis 45 microhomogenizer and a Bronwill MSK cell homogenizer prior to centrifugation.

[b] No visible pellet was present after centrifugation at 178,000 *g*. After removal of the supernatant, the bottom of the plastic centrifuge tube was cut off and placed in a scintillation vial for counting, and was referred to as the 178,000 *g* pellet.

[c] Per cent of ^{14}C in each fraction is in comparison to that present in the initial aliquot of fragmented hyphal suspension.

In the absence of phaseollin, the hyphal tips of young *R. solani* mycelium exhibit very active protoplasmic streaming. A young hyphal tip as viewed using oil immersion and phase optics is presented in Fig. 3-A. After exposure of hyphae to phaseollin, protoplasmic streaming immediately ceased, and often the protoplast contracted from the hyphal tips (Fig. 3-B). Protoplasts assumed a granular appearance, and it became difficult to recognize internal organ-

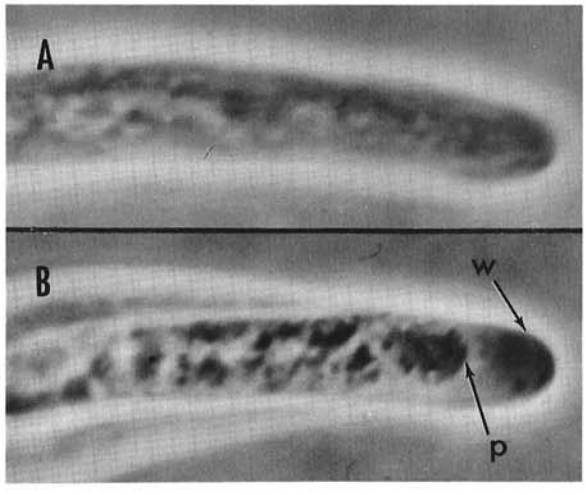

Fig. 3. Phase contrast photomicrographs of *Rhizoctonia solani* hyphae before and after exposure to phaseollin. **A)** Young hyphal tip immersed in glucose-asparagine medium supplemented with 0.5% ethanol. **B)** Young hyphal tip 1 min after exposure to phaseollin (w = hyphal wall; p = protoplast).

elles. All of these effects occurred rapidly, and were observed within 1 min after addition of phaseollin to the hyphae.

The response of *R. solani* to phaseollin appeared to be more drastic in young hyphal tips, but the same effects were observed, though less drastically, in older portions of the hyphae. In most cases the intact protoplasmic mass within a hypha contracted, but occasionally there appeared to be a general breakdown of the protoplast.

Lysis of erythrocytes by phaseollin.—It was observed initially that phaseollin induced lysis of human erythrocytes. Avine erythrocytes prepared using the technique of Kinsky et al. (19) were used for more detailed studies of hemolysis. Erythrocytes were obtained by centrifuging fresh blood (30 ml) at 2,000 *g* for 10 min at 5 C. The pellet was resuspended in 30 ml of 0.9% NaCl in 0.05 *M* potassium phosphate buffer at pH 6.8 and recentrifuged. This procedure was repeated twice. A standard suspension of erythrocytes was prepared by adding 2.0 ml of the pellet to 10 ml of the buffered NaCl solution. Phaseollin was prepared in buffered NaCl solution at various concentrations; then 24.5 ml were added to 125-ml Erlenmeyer flasks. Aliquots of 0.5 ml of the blood cell standard were added to these flasks and shaken at 100 strokes/min at 37 C. The amount of erythrocyte lysis was determined by removing 2.0-ml samples from the flasks at intervals, centrifuging them for 10 min at 2,000 *g*, and determining the hemoglobin present in the supernatant solution spectrophotometrically at 577 nm. Per cent hemolysis was determined by a comparison of the absorbance obtained to that obtained when the erythrocytes were completely lysed. Complete lysis was accomplished by adding 0.5 ml of the erythrocytes standard to 24.5 ml of a hypotonic solution (0.05 *M* potassium phosphate buffer without NaCl); after centrifugation, the absorbance of this preparation at 577 nm was ca. 0.9. Phaseollin (23 μg/ml or greater) caused a rapid and almost complete lysis of avine erythrocytes (Fig. 4).

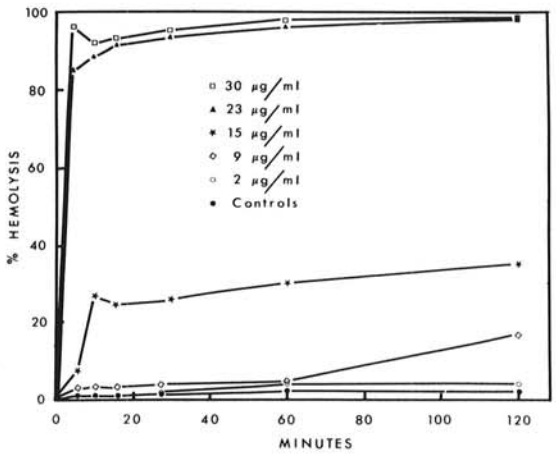

Fig. 4. Lysis of sheep red blood cells by phaseollin. Ethanol controls and no-ethanol controls gave the same readings, and are referred to as controls in the figure. Each data point represents an average of two samples.

Effect of phaseollin on bean tissue.—The effect of phaseollin on respiration (endogenous and exogenous) of hypocotyl tissue and on the release of previously incorporated $^{86}Rb^{+}$ from hypocotyl tissue were used as indicators of a response by bean plant tissue to phaseollin.

Respiratory measurements were made as with *R. solani*, except that measurements involving bean tissue were made in the dark. Respiratory rates reported are the average of three replicates of each treatment measured at 15-min intervals for 2 hr. For endogenous respiratory measurements, 0.5 g fresh wt (ca. 30 mg dry wt) of bean hypocotyl discs were added to 4.0 ml of 0.05 *M* potassium phosphate buffer at pH 6.5. Exogenous respiration was estimated in buffer which contained 1% glucose.

To incorporate $^{86}Rb^{+}$, bean hypocotyl discs (2.5 g fresh wt) were shaken (100 strokes/min) at room temperature in 10 ml of water containing 157 μc $^{86}Rb^{+}$ (6.5 μc $^{86}Rb^{+}$/μmole RbCl, New England Nuclear, Boston, Mass.) for 4 hr. The discs were then washed with six 30-ml portions of 0.05 *M* sodium phosphate buffer at pH 6.5. Twenty discs (ca. 10 mg dry wt) were added to 4.0 ml of the same buffer in 25 ml Erlenmeyer flasks and shaken at 100 strokes/min at 28 C. After the addition of phaseollin, 0.2-ml samples were withdrawn at various times, and the amount of $^{86}Rb^{+}$ released from the tissue was determined.

Phaseollin (47 μg/ml) had little or no effect on the endogenous or exogenous respiratory rates of bean hypocotyl tissue. The respiratory rate in the absence of external glucose was 254 μliters O_2/g (fresh wt)/hr for the no-ethanol control, 256 for the ethanol control, and 269 for the phaseollin-treated sample. In the presence of external glucose, the rate was 324 for the no-ethanol control, 325 for the ethanol control, and 326 for the phaseollin-treated tissue. Phaseollin at the same concentration did cause a rapid release of $^{86}Rb^{+}$ from hypocotyl tissue into which this cation had previously been incorporated (Fig. 2-D). However, the concentration of phaseollin per gram of bean tissue was approximately 3 times as great in the leakage studies as in the respiration studies. Phaseollin does, however, have the potential to induce a physiological response in bean tissue.

Growth of R. solani in the presence of cholesterol and phaseollin.—Phaseollin induces many of the same physiological responses as does another group of antifungal antibiotics, the polyenes (18, 21). It is possible to nullify the inhibitory action of the polyene antibiotics by additions of sterols to the growth medium. Additions of cholesterol (50 μg/ml) to the standard growth medium did not prevent the inhibitory effect of phaseollin (15 μg/ml) on *R. solani*, however (Table 3).

DISCUSSION.—Müller (24) reported in 1956 that, when a conidial suspension of *Monilinia fructicola* was added to the seed cavities of detached pods of French bean, the "diffusates" from the pods had antifungal activity. When he placed zoospores of

TABLE 3. Growth of *Rhizoctonia solani* in the presence of cholesterol and phaseollin

Treatment[a]	*R. solani* growth[b]	
	mg (dry wt)/flask	% of control
No ethanol control	17.3	
Ethanol control	15.8	91
Cholesterol (50 μg/ml)	15.3	88
Phaseollin (15 μg/ml)	7.0	40
Cholesterol (50 μg/ml) and phaseollin (15 μg/ml)	6.9	39

[a] Phaseollin and cholesterol (Sigma Chemical Company, St. Louis, Mo.) were added to 24-hr-old cultures of *R. solani* by first dissolving them in 95% ethanol, then adding the ethanol solution to buffered medium. The final concentration of ethanol in all treatments except the no-ethanol control was 0.5%.

[b] The cultures were incubated for 24 hr after treatment. Cultures containing no ethanol served as controls. Each value represents the mean of four replicates.

Phytophthora infestans in these diffusates, the zoospores would often swell and burst within 60 sec (24, 25). He proposed, therefore, that the phytoalexin present in the diffusates from bean pods had an effect on the zoospore membrane. Since then, it has been shown that phaseollin is present in such bean pod diffusates (4, 26), but that at least one other antifungal compound is also present (28). Hence, it is not known whether Müller observed the effect of phaseollin or of some other compound or of a mixture of antifungal compounds.

Disruption by phaseollin of any of a number of processes occurring in rapidly growing cells of *R. solani* could initiate a chain of events that might result in some of the effects which we have observed. Our observations, however, support the hypothesis that the plasma membrane or some process necessary for membrane function is affected by one of the phytoalexins of bean. Disruption of the plasma membrane could explain leakage of material from hyphae and the decrease in the dry weight of the fungal mycelium on exposure to phaseollin. The ability to actively accumulate glucose would also be reduced. Rapid lysis of erythrocytes by phaseollin is also consistent with the membrane disruption hypothesis.

The hydrophobic character of phaseollin suggests that it might associate with lipid material within fungal hyphae. Although the cell fragmentation experiment performed in this study has many limitations, the results obtained are consistent with the hypothesis that the plasma membrane is the primary site of "binding" of phaseollin. Most of the phaseollin was associated with the hyphal fraction removed by centrifugation at 500 *g* for 15 min. This fraction should contain the large plasma membrane fragments. Of course, many other components of the hyphae, such as the cell wall and nuclei, are also present in this fraction.

The type of respiration of *R. solani* observed in the presence of phaseollin may be due indirectly to membrane damage. Although inhibition of exogenous respiration could be explained by inhibited substrate uptake, it is more difficult to explain why endogenous respiration is stimulated by phaseollin. When an external carbon source such as glucose was added to starved mycelium, there was a marked stimulation of O_2 uptake (Fig. 2-B). It is unlikely that stimulation of O_2 uptake in the starved mycelia by phaseollin was due to utilization of phaseollin as a carbon source, since it was shown that *R. solani* does not significantly degrade phaseollin during short exposures. It may be possible that the stimulation of O_2 uptake by phaseollin is due to decompartmentalization of potential respiratory substrates within the mycelia as a result of membrane damage. Alternatively, membrane damage could result in an uncoupling of oxidative phosphorylation and increased O_2 uptake.

The antibiotic action of the polyenes, another group of antifungal compounds, is the result of disruption of fungal membranes (18, 21). These antibiotics also cause leakage of cellular constituents and loss of mycelial dry weight, and they decrease the ability of the fungus to take up external substances (7, 9, 16, 18, 21). They also lyse erythrocytes (17, 19). The effect of polyenes on respiration, as in the case of phaseollin, is inconsistent; respiration is sometimes stimulated, but in other situations it is inhibited (6, 9, 18). This variation in respiratory response has been attributed to the indirect effect of these compounds on respiration as a result of fungal membrane damage (18). The respiratory system per se does not appear to be the primary site of action of phaseollin because the respiratory response of *R. solani* to phaseollin was not the same for endogenous and exogenous respiration. Also, there was little or no measurable effect of phaseollin on the respiration of bean hypocotyl tissue, even though phaseollin did cause a rapid release of $^{86}Rb^{+}$ from this tissue.

Although phaseollin and the polyene antibiotics have similar effects on cells of sensitive organisms, there are some differences. The specific components of the cell membrane with which polyenes interact appear to be sterols (18, 20, 21). When sterols are added to a growth medium containing a polyene antibiotic, the polyene binds to the sterols and the antibiotic effect of the polyene is annulled (8, 22). Organisms which do not contain sterols in their membranes, such as *Pythium* species, are not sensitive to polyene antibiotics (1, 32). When cholesterol was added with phaseollin to the glucose-asparagine medium, there was no reversal of phaseollin's inhibitory effect on *R. solani*. It was also observed that *Pythium ultimum* is inhibited by phaseollin (*unpublished data*). These observations suggest that if the site of action of phaseollin is in the membrane it is not the same as that of the polyene antibiotics.

How membrane damage of *R. solani* could initiate the microscopically observed response is not clear. Such protoplasmic reactions have been observed in other organisms in response to membrane-damaging compounds. The site of action of the toxin produced by *Helminthosporium victoriae* is believed to be the plasma membrane (31). This toxin causes cessation of protoplasmic streaming in sensitive oat protoplasts

within 10 min after exposure (31). The *H. victoriae* toxin also causes shrinking of protoplasts away from the cell walls of oat root cells (33, 38). Fungal protoplast shrinkage has occasionally been observed when fungal cells were exposed to polyene antibiotics (34). In contrast to the very rapid effect of phaseollin on *R. solani*, the time required for protoplast shrinkage observed in the above instances was much longer [about 4 hr in oat cells treated with *H. victoriae* toxin (33)].

The apparent fungistatic effect of phaseollin on *R. solani* is rather surprising in relation to the large loss of cellular constituents that it induces. It may be that a few hyphae within the mycelial pads escape exposure to phaseollin, and that these initiate new growth from the pads. We have observed an apparent fungicidal effect of phaseollin on *Fusarium solani* f. sp. *phaseoli* spores (*unpublished data*). The initiation of new growth by *R. solani* in the presence of phaseollin suggests that the fungus adapted to phaseollin. Recovery of the fungus after exposure to phaseollin is not due to evaporation of ethanol from the medium and a subsequent removal of phaseollin from solution by crystallization. Examination of the medium at the time of renewed fungal growth failed to reveal phaseollin crystals. Furthermore, when DMSO (bp 189 C) was used as the phaseollin solvent, adaptation still occurred. Preliminary studies indicate that adaptation may involve degradation of phaseollin (*unpublished data*). This type of adaptation of fungi to the other pterocarpan phytoalexins has been reported (2, 13, 14, 15, 39).

It was stated previously that one reason for studying the mode of action of a phytoalexin was to discover a way to specifically annul the phytoalexin's antibiotic effect, in order to determine the possible role of this compound in disease resistance. The likelihood that phaseollin affects the cell membrane would appear to make it very difficult to annul specifically its inhibitory effects. Despite this difficulty, however, further elucidation of the mode of action of phaseollin or any other phytoalexins should provide a means for determining the role of phytoalexins in disease resistance.

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