

Phytotoxicity of Phaseollin to, and Alteration of Phaseollin by, Cell Suspension Cultures of *Phaseolus vulgaris*

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ABSTRACT

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The effect of phaseollin on growth and viability of cell suspension cultures of kidney bean, *Phaseolus vulgaris* was determined. Phaseollin at 16 and 32 $\mu\text{g/ml}$ inhibited the growth of the cell suspension cultures. Within 30 min after treatment with 32 μg of phaseollin/ml, 99% of the cells were killed. Prior exposure to a low concentration of phaseollin (4 $\mu\text{g/ml}$) did not significantly alter the sensitivity of the cultures to the higher concentrations (32 $\mu\text{g/ml}$) of phaseollin. Exogenously added phaseollin (4 $\mu\text{g/ml}$) had a half-life of approximately 4 hr in cell suspension cultures of *P. vulgaris*.

Additional key words: phytoalexins, pterocarpan, isoflavonoids.

When ^{14}C -labeled phaseollin was added to the cultures, ^{14}C appeared in an ethyl acetate-and H_2O -insoluble product(s) associated with cell debris. No ^{14}C -labeled phaseollin-like compounds accumulated in significant quantities. Phaseollin was not produced by mung bean, *Phaseolus aureus* tissue but exogenous phaseollin disappeared from cell suspension cultures of this species. Therefore, the ability to metabolize phaseollin may not be associated specifically with species that readily produce phaseollin.

Tissues of *Phaseolus vulgaris* L. produce the pterocarpanoid phytoalexin phaseollin in response to various stimuli (23, 31), and high concentrations can accumulate in fungus-infected tissue (3, 26, 32). Phytoalexins have been regarded as products of necrobiosis of the host cell (19), but it is still unresolved whether phaseollin is such a product (2, 12, 20, 23, 24). The *in vitro* antifungal activity of phaseollin is well documented (3, 12, 29, 30). Previous studies have demonstrated that phaseollin can cause permeability changes in *P. vulgaris* tissue (15, 30), but little is known about its phytotoxicity. Studies on the possible necrobiotic origin of phaseollin could be complicated if it is toxic to *P. vulgaris*.

Phytoalexins also have been considered to be end products of a host-parasite interaction (9). Studies on phaseollin and other isoflavonoid phytoalexins have demonstrated that various fungi can metabolize these compounds to different products, and there is tentative evidence that such alterations may occur *in vivo* as well as *in vitro* (32). It has not yet been demonstrated conclusively whether phaseollin also is metabolized by *P. vulgaris* tissue, but several observations suggest that phaseollin metabolism (turnover) may occur in this tissue. After phaseollin production had been induced by an abiotic stimulus, phaseollin level in the tissue decreased (23). In addition, several isoflavonoids that may be synthesized from phaseollin have been isolated

from *P. vulgaris* tissue (31). Several studies (4, 5) have demonstrated that other isoflavonoids previously thought to be end products undergo further metabolism by plant tissue. Results of a recent study by Dewick (13) suggest that the pterocarpanoid phytoalexins medicarpin and maackiain are subject to turnover in *Trifolium pratense*.

The purposes of the present study were: (i) to determine whether exogenously added phaseollin is toxic to *P. vulgaris* tissue and (ii) to determine whether *P. vulgaris* metabolizes exogenously supplied phaseollin. Preliminary studies on the possible metabolism of phaseollin by mung bean (*P. aureus*) also were conducted.

MATERIALS AND METHODS

Cell suspension cultures.—Cell suspension cultures of kidney bean, *P. vulgaris* L., originally derived from root tissue, were obtained from M. T. Lieberman, Dept. of Agronomy, Cornell University. These were grown in 125-ml Erlenmeyer flasks in the dark at 28 C with shaking at 220 rpm. Subcultures were made every 14-17 days by transferring 10 ml of the cell suspension (approximately 20 mg, dry wt), avoiding large aggregations, to 40 ml of fresh SCP medium (22).

Cell suspension cultures of mung bean, *P. aureus* Roxb., also derived from root tissue, were obtained from M. T. Lieberman and were grown under the same conditions except that subculturing was done every 14 days by transferring 9.0 ml of cell suspension (approximately 60 mg, dry wt) to 40 ml of fresh B_5 medium (16).

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Growth rates of both cell suspension cultures were obtained by measuring the change in dry weight. The solid contents of each flask were collected on tared Whatman No. 1 filter paper by means of a vacuum filtration apparatus and the samples were weighed after drying at 75 C for 24 hr.

The cultures were checked routinely for possible contamination by visual inspection and by transferring small amounts to nutrient broth (Bacto) medium.

Production of diseased mung bean tissue and assay for phaseollin.—Mung bean seeds were obtained from a local grocery store. Seedlings were inoculated with *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyder and Hans. isolate R-10 or *Rhizoctonia solani* Kuehn isolate RB by the same procedures used previously for *P. vulgaris* (26, 32). Four wk after inoculation with *F. solani* and 4 days after inoculation with *R. solani*, *P. aureus* hypocotyls bearing the typical cortical rot symptoms of *F. solani* infections and the delimited lesions characteristic of *R. solani* infection were harvested and the infected tissues were assayed for phaseollin as described previously (26).

Phytoalexins.—Phaseollin and phaseollinisoflavan were obtained as described previously (32). Solutions of ¹⁴C-labeled phaseollin were prepared by diluting with unlabeled phaseollin the ¹⁴C-labeled phaseollin (2,857 dpm/μg) prepared from acetate-U-¹⁴C by VanEtten and Bateman (30). Quantitation of phaseollin was accomplished spectrophotometrically using the extinction coefficient in ethanol at 279.5 nm (10). Phaseollin was dissolved in dimethylsulfoxide (DMSO) and added to the suspension cultures; the final DMSO concentration was 0.6% or less.

Toxicity studies.—The effects of phaseollin on bean cell suspensions were evaluated by determining its effect on the growth rate of phaseollin-treated cells and their ability to accumulate and retain the vital stain, neutral red. For the latter assay, a modification of Tribe's procedure as described by Basham and Bateman (6) was used. One-half ml of a cell suspension culture was added to 2 ml of a solution containing 800 mM KNO₃, 1 mM CaCl₂, 20 mM phosphate buffer (pH 7.5), and 0.01% neutral red. After 30 min the cells had settled and the supernatant was removed and replaced with 2.0 ml of the same solution minus the neutral red. After an additional 30 min, the process was repeated and then a portion of the cell suspension was placed on a glass microscope slide, covered with a cover slip, and viewed at ×200. Live cells appeared dark red and were plasmolyzed, whereas dead cells appeared colorless. The percentage of live cells was determined by examining single cells and small cell aggregates and scoring 1,000 or 2,000 cells for each treatment. Controls consisted of non-treated cell suspension cultures and of those treated with the same amount of DMSO as the phaseollin-treated cultures.

Extraction and assay of phaseollin and ¹⁴C-labeled compounds from cell suspension cultures.—In all experiments except those in which ¹⁴C-labeled phaseollin was employed the following procedure was used. The cell suspension cultures were mixed with an equal volume of 95% ethanol and held at 4 C overnight or longer. Cell debris was removed by filtering the samples through Whatman No. 1 filter paper. The cell debris was washed with a few ml of 50% ethanol and the ethanol was

evaporated from the combined filtrates under reduced pressure. The resulting aqueous fraction was partitioned three times with equal volumes of chloroform and the chloroform fractions were combined and evaporated to dryness. The residue was dissolved in ethanol and spotted, along with phaseollin and phaseollinisoflavan standards, on silica-gel (250-μm) thin-layer chromatography plates (Kontes, F254, Vineland, N.J. 08360). The plates were irrigated with benzene + methanol (9:1) and, after drying, phaseollin and phaseollinisoflavan were located under UV light or by spraying the plate with diazotized p-nitroaniline (R_f of phaseollin = approximately 0.7, and R_f of phaseollinisoflavan = approximately 0.5). In certain experiments, samples were recovered from the chromatography plate by collecting the silica gel in Pasteur pipets tightly packed with glass wool and washing it several times with 1 ml of 95% ethanol. The UV absorbance spectrum of the eluent was used to quantify phaseollin and to monitor different locations on the plate for other compounds with UV spectra similar to phaseollin or other known isoflavonoids.

Bean cell suspension cultures treated with ¹⁴C-labeled phaseollin were ground with equal volumes of ethyl acetate in a Waring Blendor for 1 min. The ethyl acetate fraction was collected and the aqueous fraction (which contained the cell debris) was partitioned seven times with equal volumes of ethyl acetate. The ethyl acetate fractions were combined, dried, and chromatographed as described above. The irrigated chromatograms were examined under UV light and the UV-quenching bands and the areas between them were collected. Each silica gel sample was eluted 10 times with 2 ml of ethanol as described above. In some experiments, the UV absorbance spectrum of the filtrate was monitored. The total filtrate then was transferred to scintillation vials and the ethanol was evaporated. Ten ml of scintillation fluid [toluene + 0.5% 2,5-diphenyloxazole + 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene] was added and the samples were assayed for ¹⁴C.

The radioactivity in the aqueous fraction and the cell debris was determined as follows. A 5-ml sample of the aqueous fraction (of 50-55 ml total) was centrifuged at 2,000 g for 10 min and the supernatant liquid was transferred to scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, MA 02118) scintillation fluid. The pellet containing the cell debris was suspended in 5 ml of H₂O and the suspension was added to scintillation vials containing 10 ml of Aquasol.

All ¹⁴C samples were assayed with a Beckman LS 355 liquid scintillation spectrometer and the counts per minute values were converted to disintegrations per minute (dpm) by the external standard method of quench correction. The ¹⁴C data are expressed on a dpm per flask basis.

RESULTS

Under the incubation conditions used in this study, bean cell suspension cultures reached a phase of rapid growth in 5-6 days (Fig. 1). Therefore, for most of the following experiments 5.5-day-old cell suspension cultures were used.

Phaseollin production by cell suspension cultures and diseased mung bean hypocotyls.—No phaseollin was

detected in 5- or 14-day-old cell suspension cultures of kidney bean. Since previous studies (14, 21) have demonstrated that CuCl_2 induces phytoalexin production in plant tissue, 2.0 ml of a 1mM CuCl_2 solution was added to 5-day-old cell suspension cultures of kidney bean. Phaseollin was readily detected in these cultures 24 hr later.

Attempts to detect phaseollin in *F. solani*- and *R. solani*-infected mung bean hypocotyls were unsuccessful even though these tissues contained numerous compounds that reacted positively with diazotized p-nitroaniline on TLC plates. No phaseollin was detected in mung bean cell suspension cultures before or after treatment with CuCl_2 as described above.

Phytotoxicity.—Phaseollin was added to 5.5-day-old cell suspension cultures of kidney bean at 4, 16, and 32 μg of phaseollin/ml of medium (final DMSO concentration = 0.3%). Sixty hr later, dry weight measurements were taken. The DMSO control and the low phaseollin treatment (4 $\mu\text{g}/\text{ml}$) caused a slight decrease in growth compared to that of nontreated tissues (Fig. 2-A). Phaseollin at 16 $\mu\text{g}/\text{ml}$ and 32 $\mu\text{g}/\text{ml}$ caused a pronounced inhibition of growth. The dry weight of the 32 $\mu\text{g}/\text{ml}$ treatment was less than the dry weight of the tissues at the beginning of the experiment at 5.5 days, suggesting that many, if not all, of the cells were dead.

Certain fungi become tolerant of high levels of phaseollin if they are first exposed to low levels of the phytoalexin

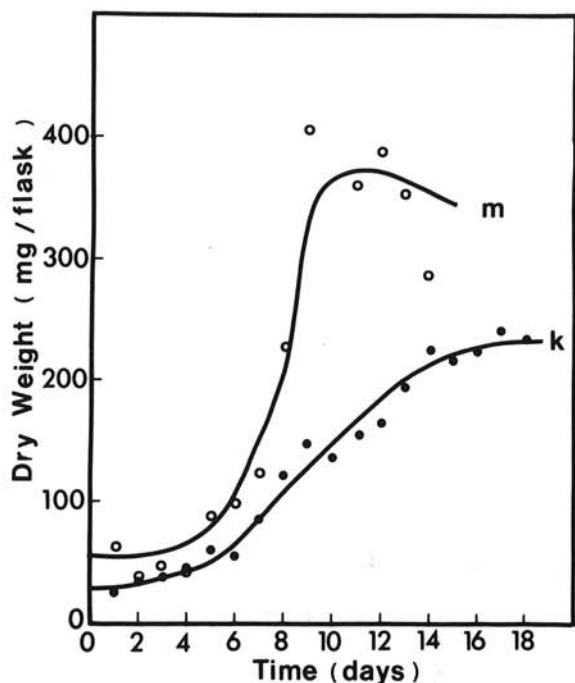


Fig. 1. Growth curves (as mg dry wt/flask) of cell suspension cultures of kidney bean, *Phaseolus vulgaris* (k) and mung bean, *P. aureus* (m). Dry weight changes were determined by filtering the contents of 1 flask (approximately 50 ml) each day and drying the tissue at 75 C for 24 hr. Each datum point for kidney bean represents the average of three experiments; those from mung bean are from one experiment.

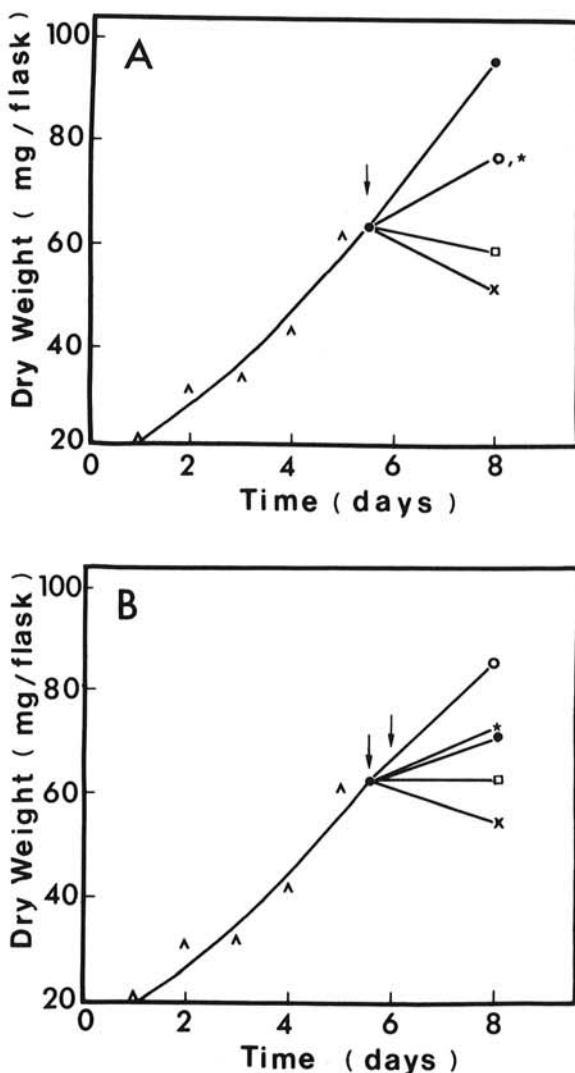


Fig. 2-(A, B). Effect of phaseollin on the growth of kidney bean cell suspension cultures which have not A) or which have B) been pretreated with 4 μg of phaseollin/ml. Growth (as milligrams dry wt/flask) was recorded for 5.5 days (\blacktriangle), then cultures were treated. In A) phaseollin, at a final concentration of 4 $\mu\text{g}/\text{ml}$ (*), 16 $\mu\text{g}/\text{ml}$ (\square), and 32 $\mu\text{g}/\text{ml}$ (x) of medium, was added to the cultures at the time indicated by the arrow, and the cultures were harvested after an additional incubation period of 60 hr. Controls consisted of cultures receiving neither phaseollin or DMSO (\bullet) and cultures receiving the same amount of DMSO (o) as used for the phaseollin treatments (final DMSO concentration = 0.3%). The datum for 16 $\mu\text{g}/\text{ml}$ is the average of three flasks and the datum for 32 $\mu\text{g}/\text{ml}$ is an average of two flasks. All other data points represent the average of four flasks from two experiments. In B) a final concentration of 4 μg of phaseollin/ml was added to the cultures at the time indicated by the first arrow and 12 hr later the cultures were supplemented (second arrow) with the same concentrations of phaseollin as used in A). After an additional 48 hr the cultures were harvested. Controls consisted of nontreated cultures (\bullet) and cultures (o) receiving two DMSO treatments (final DMSO concentration = 0.3% in each treatment) at the same time cultures received the phaseollin treatments. All data points are an average of four samples from two experiments.

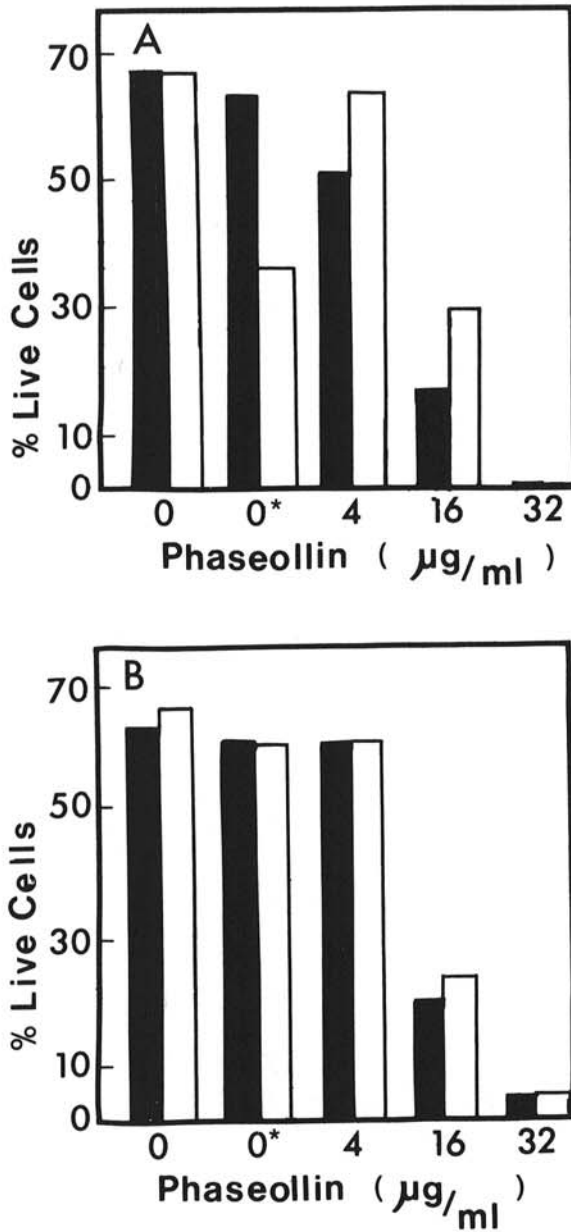


Fig. 3-(A,B). Toxic effect of A) a 30-min and B) a 60-hr exposure to phaseollin on kidney bean cell suspension cultures which were or were not pretreated with a low concentration of phaseollin. Toxicity was determined by the ability of cells to retain the vital stain, neutral red. Cultures 5.5-day-old were administered phaseollin at a final phaseollin concentration of 4, 16, and 32 µg/ml (solid bars) or 5.5-day-old cultures were treated with 4 µg of phaseollin/ml (final concentration) and 12 hr later the medium was supplemented with 4, 16, and 32 µg of phaseollin/ml (open bars). The cultures were assayed A) 30 min or B) 60 hr after the final phaseollin treatment. Controls consisted of no treatment (o) or DMSO (*) treatment (solid bars) are data on cultures that received a single 0.3% DMSO treatment and open bars are data of cultures that received two sequential 0.3% DMSO treatments). The percentage of live cells was determined by assaying 2,000 cells/treatment in A) and 1,000 cells/treatment in B).

(27, VanEtten and Stein, *unpublished*). Associated with tolerance there is often an enhanced ability to catabolize phaseollin. To determine if kidney bean cell suspension cultures would develop tolerance to phaseollin, 5.5-day-old cultures were treated with 4 µg of phaseollin/ml and then 12 hr later were treated with an additional 4, 16, or 32 µg of phaseollin/ml. Pretreatment with phaseollin did not substantially change the growth response of kidney bean cell suspension cultures to phaseollin (Fig. 2-B).

To determine the effect of phaseollin on cell viability, 5.5-day-old kidney bean cell suspension cultures were exposed to the same series of treatments as above and incubated for 30 min (Fig. 3-A) or 60 hr (Fig. 3-B) before they were assayed with neutral red. Essentially the same percentage of cells was dead whether the tissue was assayed 30 min or 60 hr after the final phaseollin treatments indicating that toxic action of phaseollin was rapid. As with the growth rate studies, pretreatment of the cell suspension with a low concentration of phaseollin did not render the cells tolerant to the highest concentration of phaseollin (32 µg/ml) supplied later (Fig. 3-A and 3-B). The response of cell suspension cultures to two subsequent DMSO treatments sometimes was variable (e.g., Fig. 2-B vs. Fig. 3-A), and because of this it is not possible to say whether the apparent small enhanced tolerance to 4 µg and 16 µg of phaseollin in Fig. 3-A is real or not.

Alteration of phaseollin by bean suspension cell cultures.—In preliminary studies 4 µg of nonlabeled phaseollin/ml (final DMSO concentration = 0.3%) were added to 5.5-day-old cell suspension cultures of both kidney and mung bean cells. Controls consisted of autoclaved cultures (1. kg/cm² for 20 min), nontreated cultures, and DMSO treated cultures. After 2, 4, 8, 20, 32, 53, 68, and 90 hr one-half of the contents of each flask was assayed semi-quantitatively by estimating phaseollin concentration from the intensity of the color that developed at the *R_f* coincident with phaseollin when the TLC plates were sprayed with diazotized p-nitroaniline. No phaseollin was detected in the nontreated or DMSO treated controls. Phaseollin concentration in the autoclaved cultures appeared to remain unchanged throughout the 90-hr incubation period. After 52 hr, phaseollin was not detected in the mung bean suspension cell cultures and only a small amount was detected after 90 hr in the kidney bean cell suspension cultures. Thus, both mung bean and kidney bean cell suspension cultures transform phaseollin to chloroform-insoluble substance(s).

Four experiments were done with ¹⁴C-labeled phaseollin and although various modifications were tried the results were similar to the representative experiment described below.

In an attempt to enhance the metabolism of phaseollin, nonlabeled phaseollin (final concentration, 4 µg/ml) was added to 5.5-day-old cultures 12 hr before addition of the ¹⁴C-labeled phaseollin (55 dpm/µg). A total of 11,700 dpm of ¹⁴C-labeled phaseollin was added to each flask (final concentration of ¹⁴C-labeled phaseollin equal 4 µg/ml). As a control, autoclaved cultures were treated similarly except that the pretreatment with unlabeled phaseollin was omitted. One flask per treatment was assayed at 1/4, 4, 31, 60, and 90 hr. The amount of phaseollin detected in the autoclaved cultures was fairly constant throughout the 90-hr period (Fig. 4-A and B).

Most of the ^{14}C recovered from the autoclaved cultures was present at the R_f of phaseollin on thin-layer chromatograms. For example, in the 90-hr sample 80% of the ^{14}C was recovered as phaseollin (7,987 dpm), 9% was detected on other areas of the TLC plate (974 dpm), and 10% was detected in the combined aqueous fraction and cell debris (1,004 dpm). In contrast, the total amount of phaseollin (Fig. 4-A) and the ^{14}C -labeled phaseollin (Fig. 4-B) decreased rapidly in the nonautoclaved cultures. By 4 hr a substantial amount of the ^{14}C label was associated with the cell debris and by 60 hr most of the ^{14}C was recovered in the cell debris (Fig. 5).

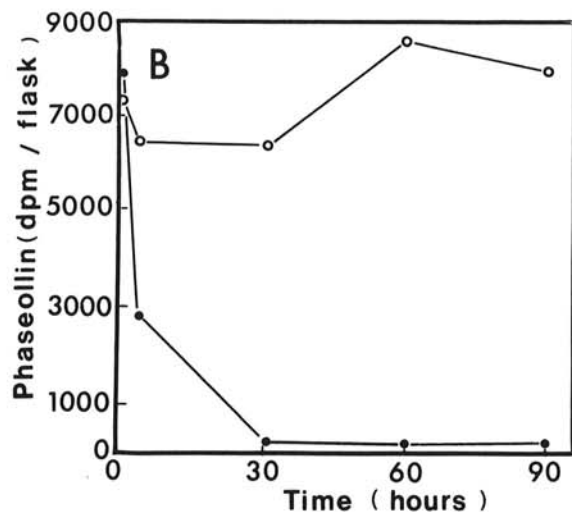
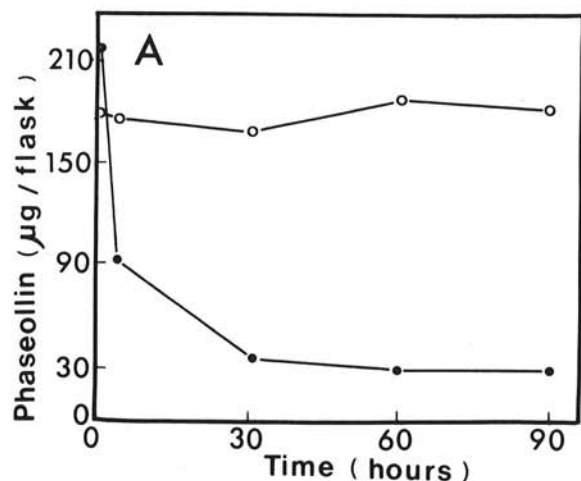


Fig. 4-(A,B). Disappearance of A) phaseollin, and B) ^{14}C -labeled phaseollin from kidney bean cell suspension cultures. Cultures 5.5 days old were treated with $4\ \mu\text{g}$ of phaseollin/ml of medium (total of $200\ \mu\text{g}$) and 12 hr later (time 0) treated with the same concentration of ^{14}C -labeled phaseollin (●) (total dpm added = $11,700/\text{flask}$). Final DMSO concentration in each treatment was 0.3%. The control (o) consisted of an autoclaved 6-day-old culture which was treated only with the ^{14}C -labeled phaseollin. The first data points are from cultures harvested 15 min after the final treatment. Each datum point is the value for one flask.

To ensure that the ^{14}C associated with the cell debris was not nonextracted phaseollin, the remaining cell debris from the 60-hr sample was refluxed with boiling ethanol, a treatment that does not degrade phaseollin. After refluxing for 22 hr, all the ^{14}C still was associated with the insoluble cell debris.

Throughout the course of this experiment, phaseollin was the only identifiable ^{14}C compound that was detected in ethyl acetate extracts of nonautoclaved cultures. For example, 64% ($2,854\ \text{dpm}$) of the total ^{14}C activity ($4,457\ \text{dpm}$) in the ethyl acetate fraction was detected as phaseollin in the 4-hr sample and 36% ($1,603\ \text{dpm}$) was distributed throughout the other areas of the chromatogram. In this experiment, and in all the other experiments with ^{14}C -labeled phaseollin, no specific area on the TLC plate ever contained much ^{14}C above that contained on chromatograms of the comparable autoclaved controls; occasionally areas at approximately $R_f 0.5$ had 100 to 350 dpm above those of controls, and after long incubation periods the origin had 350 to 400 dpm above those of controls. Although the activity detected at an R_f of approximately 0.5 occasionally was associated with materials that had UV spectra similar to phaseollin, this was not consistently observed and, as mentioned above, the amount of activity associated with such areas was always very low. The procedures used in this study would not have detected $^{14}\text{CO}_2$. However, the sum of the ^{14}C located in the cell debris, ethyl acetate, and aqueous fractions remained constant (approximately $9,000\ \text{dpm}$) in both the autoclaved and nonautoclaved cultures throughout the experiment, indicating that a significant amount of $^{14}\text{CO}_2$ was not liberated.

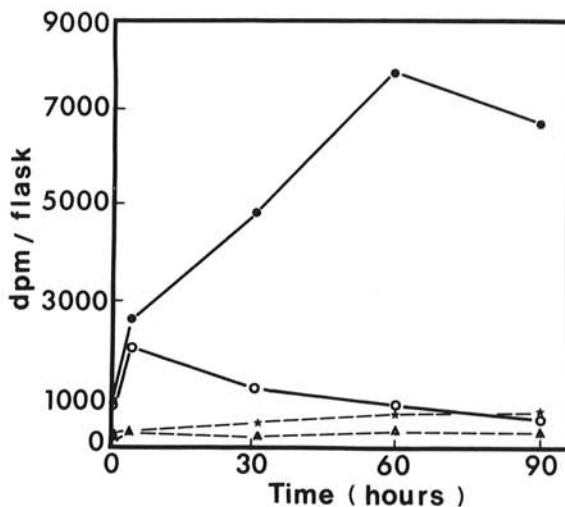


Fig. 5. Distribution of ethyl acetate insoluble ^{14}C in kidney bean cell suspension cultures after exposure to ^{14}C -labeled phaseollin. Cultures are the same as those described in Fig. 4. The ^{14}C remaining in the aqueous fraction of nonautoclaved cultures (●) and autoclaved cultures (▲) and ^{14}C remaining in H_2O -insoluble cell debris of nonautoclaved cultures (●) and autoclaved cultures (o) after extraction $\times 7$ with equal volumes of ethyl acetate. The first data points are from cultures harvested 15 min after the final phaseollin treatment. Each datum point is the value for one flask.

DISCUSSION

Our results revealed that cell suspension cultures of *P. vulgaris* are as sensitive to phaseollin as are many fungi (3, 12, 29, 30). When our results with bean suspension cultures are compared with studies (30, and VanEtten and Stein, *unpublished*) in which the antifungal activity of phaseollin was bioassayed in shake cultures at similar phytoalexin concentrations, ratio of phaseollin per milligram dry weight of the test organism, and growth conditions, approximately 16 $\mu\text{g}/\text{ml}$ inhibited both bean suspension cultures and fungi; in both cases higher concentrations result in dry weight losses. The pterocarpanoid phytoalexin pisatin produced by pea, *Pisum sativum*, also is phytotoxic. Pisatin retards the growth of primary roots of wheat (11), is inhibitory to the growth of pea callus cultures (1), and is toxic to stem epidermis cells of pea and to protoplasts prepared from pea leaf epidermis tissue (25). The sensitivity of bean cell suspension cultures to phaseollin applied exogenously may not be indicative of the sensitivity of *P. vulgaris* to phaseollin in situ as compartmentalization or other factors may influence its activity in situ. However, similar reservations can be expressed about the significance of in vitro bioassays of fungi in the interpretation of the in vivo response of fungi.

Although phaseollin potentially is as toxic to *P. vulgaris* tissue as it is to many fungi, *P. vulgaris* tissue apparently lacks the ability to develop a tolerance to phaseollin (Fig. 2 and Fig. 3). It has been demonstrated that at least one bean pathogen can develop a tolerance of phaseollin (27, and VanEtten and Stein, *unpublished*). It is tempting to speculate that the very high concentration of phaseollin that accumulates in infected tissue is toxic to the host cells but not to certain pathogens. Shiraishi et al. (25) have suggested that the wilting associated with *Erysiphe pisi* DC. infection of pea actually may be caused by host produced pisatin.

Although no product was identified, exogenously added phaseollin was altered by cell suspension cultures of *P. vulgaris* and the half-life of exogenously added phaseollin (approximately 4 hr) was shorter than that determined for some other isoflavonoids (5). Most of the phaseollin was converted into ethyl acetate-, water-, and hot ethanol- insoluble product(s) associated with cell debris. The solubility properties suggest that this product is associated with or is a part of a macromolecule such as a polysaccharide or lignin.

It has previously been speculated that pterocarpan might serve as precursors for the biosynthesis of 2'-hydroxyisoflavans in higher plants (33). The occurrence of phaseollinisoflavan (a 2'-hydroxyisoflavan) in infected tissue together with phaseollin (a pterocarpan) suggests that phaseollin may be a precursor of phaseollinisoflavan. In addition, some fungi cleave the benzylphenyl linkage in phaseollin, converting it to phaseollinisoflavan (17). Analogous enzymes also may exist in *P. vulgaris* tissue, but no ^{14}C compound was observed in our experiments that definitely could be identified as phaseollinisoflavan.

One possible reason for the failure to detect phaseollinisoflavan may be that the metabolic pathways of exogenously added phaseollin, especially when added with a carrier solvent such as DMSO, are different from

those involved in endogenous phaseollin synthesis and turnover. It has previously been demonstrated that exogenously added isoflavonoids can participate in both anabolic and catabolic pathways (5). The preliminary results with cell suspension cultures of mung bean suggest that catabolic pathways for phaseollin may exist in the absence of anabolic pathways. Mung bean cells apparently do not synthesize phaseollin but they do alter phaseollin. Experiments with cell suspension cultures of *P. vulgaris* indicate that phaseollin synthesis and catabolism (turnover?) may be occurring simultaneously. A comparison of the specific activity of phaseollin at the start of the experiment (55 dpm/ μg) to that at 15 min (36 dpm/ μg), 4 hr (31 dpm/ μg), and 30 hr (7 dpm/ μg) (Fig. 4-A vs. 4-B) reveals that it decreased steadily, suggesting de novo synthesis of phaseollin. Phaseollin may induce the synthesis of phaseollin. However, the amount of phaseollin recovered in the 15-min sample (Fig. 4-A) was greater than the amount of ^{14}C -labeled phaseollin added at "time 0", indicating that some phaseollin must have been present in these cultures at "time 0". The decrease in the specific activity of the total extractable phaseollin might have occurred because some of the nonlabeled phaseollin added as a pretreatment was retained in a metabolically inaccessible site (e.g., dead cells) and the added ^{14}C -labeled phaseollin was metabolized preferentially.

Recently, several researchers (8, 14) suggested that the pterocarpan to 2'-hydroxyisoflavan transformation is not the likely origin of 2'-hydroxyisoflavans. If this is true then we would not expect *P. vulgaris* to readily transform phaseollin to phaseollinisoflavan. Based on chemical oxidation studies, Cornia and Merlini (8) have suggested that 2'-hydroxyisoflavans might serve as the precursors for pterocarpan. With the use of ^{14}C -labeled precursors, Dewick and Martin (14) demonstrated that pterocarpan and isoflavans can be interconverted in *Medicago sativa*. They suggested a common intermediate precursor (possibly an isoflav-3-ene) for both pterocarpan and 2'-hydroxyisoflavans. In this regard, it is interesting that Kinoshita et al. (18) have isolated an isoflav-3-ene (glabrene) that has aromatic ring substitution patterns identical to those of phaseollin and phaseollinisoflavan.

Studies on fungal-phaseollin interactions have indicated two other possible means of fungal catabolism of phaseollin in addition to conversion to phaseollinisoflavan. Phaseollin can be metabolized to 1a-hydroxy-phaseollone or 6a-hydroxyphaseollin (7, 28). In addition, the structurally-related compound phaseollidin is found to occur along with phaseollin in bean tissue (2). Although any of these compounds conceivably might be intermediates in the metabolism of phaseollin by *P. vulgaris* tissue, all these compounds are soluble in ethyl acetate and should have been detected readily in the analysis system employed. We found no evidence, however, for the occurrence of these compounds as intermediates in phaseollin metabolism by *P. vulgaris*.

Previous suggestions that phytoalexins represent end products specifically elaborated in infected tissue (9) may be reinterpreted as abnormally high accumulations of isoflavonoids resulting from their phytotoxicity. Our results indicate that exogenously supplied phaseollin can be converted to ethyl acetate-insoluble products and that phaseollin is toxic to kidney bean cell suspension cultures.

It is therefore tempting to speculate that microbial infection induces enhanced pterocarpan synthesis. The greater production of these compounds may result in increased phytotoxicity and inactivation of the phaseollin metabolizing system. Clarification of why high levels of phaseollin are present in infected tissue therefore requires further elucidation of normal isoflavonoid biosynthesis and turnover and greater understanding of the mechanisms by which phaseollin production is induced by microorganisms.

Note: Since the acceptance of this manuscript, the results of a similar study have appeared in Skipp, R. A., C. Selby, and J. A. Bailey. 1977. Toxic effects of phaseollin on plant cells. *Physiol. Plant Pathol.* 10:221-227.

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